Universidade de Aveiro Departamento de Biologia
Ano 2016

JESSICA CRISTINA LUGO LADEWIG

EFEITOS DE POLUENTES EM PARAMETROS FISIOLÓGICOS DE BIVALVES EM DIFERENTES LOCAIS DO HEMISFÉRIO NORTE

EFFECTS OF POLLUTANTS ON PHYSIOLOGICAL PARAMETERS OF BIVALVE SPECIES IN DIFFERENT LOCATIONS OF THE NORTHERN HEMISPHERE

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Ciências do Mar e do Ambiente, realizada sob a orientação científica do Doutor Fernando Manuel Raposo Morgado, Professor Associado do Departamento de Biologia da Universidade de Aveiro e co-orientação do Doutor Jaime Rendón von Osten, Professor e Investigador do Centro de Ecologia, Pesca e Oceanografia do Golfo do México da Universidade Autónoma de Campeche.

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio.

É um paradoxo a Terra se mover ao redor do Sol e a água ser constituída por dois gases altamente inflamáveis. A verdade científica é sempre um paradoxo, se julgada pela experiência cotidiana que se agarra à aparência efêmera das coisas.

Karl Marx

Àquele que me ensinou a andar, falar e nunca desistir dos meus sonhos. Tu foste a minha maior inspiração não só para terminar esta tese como para seguir os caminhos mais tortuosos dessa vida em busca de um objetivo. Obrigado por ter me ensinado tanto e por ter sido o meu porto seguro durante 30 anos. A homenagem é singela, mas o meu amor por ti é eterno e gigantesco.

Rainer Kurt Erich Ladewig
29/06/1946 - 13/01/2013

o júri

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Agradecimentos

Agradeço a todas as pessoas que, direta ou indiretamente, ajudaram na realização deste trabalho:

À Fundação da Ciência e Tecnologia (FCT) pela bolsa de estudos (SFRH/BPD/62561/2009) e apoio financeiro ao longo deste trabalho.

Aos meus orientadores Fernando e Jaime por acreditarem em mim e nas minhas capacidades como pesquisadora, por terem tanta paciência comigo durantes esses anos e por me incentivarem nos momentos mais dificies da minha tese.

À grande equipa do México (Jaime, Martin Memije, Alejandro Ortiz Arana), que me ajudaram com todas as saídas de campo, análises de laboratório e tudo mais que precisei.

Ao seu Aldiro por todas as saídas de campo e corbículas “pescadas”. Ao Abel por todo apoio dentro do laboratório, essencial para o cumprimento dos meus objectivos. A malta do Letal, especialmente a Cátia Venâncio (obrigada pelas colaborações, amizade e aprendizado), ao Emanuele Fasola (obrigada pela amizade e pela inesquecivel acolhida italiana) e à Fátima (obrigada pelas correções, pela amizade e pela companhia nos almoços da UA).

Rhaul (sim, você merece um parágrafo só seu), você nesses anos se tornou um dos meus melhores amigos, meu irmão aveirense. Você me mostrou que ser megalomaniaco no trabalho (como nós somos) pode sim trazer coisas boas, me mostrou o sentido do verdadeiro autruísmo e da verdadeira amizade. Obrigada pelas conversas, por me ajudar mais do que você tem noção (apesar que hoje em dia, eu tenho certeza que tu sabe), por todas a noites doidas na praça (não quebraria um dedo por ninguém mais além de ti!), pelas parcerias, pelos ensinamentos, pelas viagens, aventuras, enfim, por tudo!!! E que venham novas colaborações, viagens e doideras! Amo você!!

Thayres e Thibaud, meu casal favorito de todos os tempos, com vocês eu aprendi tanto e nunca me senti tão amada por duas pessoas. Obrigada pelo apoio sem limites, pela amizade sincera, pelo amor, pelas viagens, pelos perrengues, pelos aniversários e festas… por tudo. Espero que a gente more muito perto no Brasil, porque já não imagino minha vida sem a presença de vocês!

À todos que de uma forma ou outra morei junto: Ni, Nevena, Deisa, Mafalda, Rhaul, Carol, Paulinha, Chris, Andreia, Bruna, Alex, Magda. Cada um contribuiu de uma forma unica e significativa a minha vida. Bruna e Alex obrigada por terem me proporcionado um lar e não só um quarto, morro de saudades de vocês! E agradeço ambos por terem aguentado a barra em um dos momentos mais tortuosos da minha jornada, sem ajuda e apoio de vocês eu não sei o que faria. E aqueles que me ajudaram no meu periodo sem bolsa: Thayres, Thibaud, Rhaul, Susana, Violeta, Paulinha, Carol, Marcia, Izabel, Magda e Roberta. Não tenho palavras para agradecer a amizade, amor, carinho e zelo pelo meu bem estar.

Paulinha, meu xuxu!! Desde o começo até o fim, você foi a melhor das surpresas em minha vida! Você me trouxe um gostinho daquela convivência da graduação e se tornou uma grande amiga desde o começo. Amo a nossa amizade, todas as nossas aventuras, noitadas de Erasmus e a nossa jornada juntas! E aproveito esse momento para agradecer o Tcheco (Ondrej) também pela amizade, convivio, finos e tudo mais.

Susana e Violeta, não sei se existem palavras que expressem tudo o que vocês representam para mim. Obrigada pela amizade sincera, pelo amor, pelas porradas e por me fazerem enxergar que nem tudo estava perdido em alguns momentos mais intensos. Susana, você é sem sombra de dúvidas uma das razões por eu ter chego ao fim dessa tese! Obrigada pelas imensas correções e contribuições a minha tese, pelo curso relâmpago de escrita científica, pela amizade, pelo carinho, por me dar nas orelhas quando eu merecia, pelos finos, jantares, viagens e afins e por você ser uma parte tão importante da minha vida. Violeta, a pessoa mais fofinha do mundo! Obrigada por você ser simplesmente você, pelo apoio, carinho e incentivos tão cruciais para eu chegar até aqui. Amo muito vocês!

À malta brasileira mais fixe do mundo: Fabi, Nessa, Valdir, Pablito (e a inesquecível constância), Dani (amora mais linda do mundo, um cheiro bem grande na sua pessoa! Obrigada pela amizade e por me deixar fazer parte da sua vida, já morro de saudades!!!), Rhaul, Paulinha, Nisete, Fernanda Pitanga

(obrigada pela amizade constante, pelas aventuras e convivência diária) e Roberta (obrigada flor pela imensa amizade, amor, carinho, ajuda em todas as horas e pelas colaborações; a amizade vai ser eterna, te amo!). Especialmente a Carol que me mostrou várias maneiras de eu ser a melhor versão de mim mesma, obrigada pela convivência, pela amizade inestimável, por me ajudar e cuidar de mim em tantos momentos, Amo você!

À malta portuguesa mais fixe do mundo: Anita, Rita Bicho, Chico, Sofia e Marco (obrigada pelas contribuições e artigos juntos, adorei trabalhar contigo e que venham mais para o futuro!). Cada um de vocês teve um papel extremamente significativo em minha vida, obrigada pela amizade e por tudo!

Às pessoas com quem eu trabalhei junto nos períodos sem bolsa e que se tornaram parte da minha vida: Jelena, Cindy (thanks for cooking and taking so good care of me in the hell farm!), Sofia, Agneska, Izabel (obrigada pela ajuda em todas as mil tabelas, todos os nossos café e aventuras), Kaue, Tiago, Mariana, Diana, Ana Dias, Cátia.

Àqueles que me ajudaram a chegar aqui através de uma doação pelo crowndfunding (Sonia Bochnia, Lica Krug, Natalia Rodrigues, Hana Scepankova, Jelena Zivlak, Davi Vriesman, Tullus Ullus Bergamn Filho, David Foley, Ale C, Diógenes Laube, Catherine Ribeiro, Vanessa Bastos, Raquel Mello, Sara Valente, Augusto Ventura, Viktoriia Burkina, Bruno Simioni, Marcelo Amorim, Jana Vasickova, Barbara Giaccom, Irina Kuklina, Dra. Urska Demsar, Manuel Curto, Kassio Rios, Adriana Lopez, Sindy Lopez, Maikon di Domenico, Miodeli N Junior, Sidneia Lopes, Daniel Rocha e outras pessoas que não quiseram se identificar). Obrigado pela doação! Sem a grande generosidade de vocês, eu não conseguiria estar aqui nesse momento! Meu agradecimento é singelo, mas a generosidade de vocês vai estar sempre gravada no meu coração!

À minha familia pontalense, que mesmo longe esteve presente em minha vida de inúmeras formas. Especialmente a Luana, Marianna, Manoela, Daniel, Lica Krug, Laura e Paula Salge: obrigado por todas as visitas, apoio e ajuda nos momentos cruciais. Vocês moram no meu coração para sempre!

À Magda e a Izabel, tão essenciais a minha vida, não imagino ela sem vocês! Magda, tu chegou nos 45 minutos do segundo tempo mas sem teu apoio, amizade e compreensão nos últimos meses não sei se teria chego tão inteira a essa etapa. Obrigada pela convivência diária, amizade, os jantares paleo maravilhosos de todos os dias, a degustação “semanal/quase diária” de vinhos, as aventuras pelo Porto e pela vida. Amo você irmãzinha de alma maluca e que venham dez mil novas aventuras e coisas boas na nossa vida! Izabel, foram tantas aventuras e desventuras que nem sei por onde começar; obrigada pela sua presença constante em minha vida, pela amizade, por me aturar em todos os trabalhos e fora deles, por cuidar de mim nos momentos críticos, não existem palavras para expressar todo o amor e aprecio que sinto pela sua pessoa e por tudo o que passamos nesses anos. Te amo!

A malta do Crossfit Aveiro: Fabianne, Rita, Rui, Vânia, Tânia, Liliana, Zé Maria, Sandrina, Ana Peres, Sara, Sandra, Gabriela, Inês, Silvana, aos coachs Juvenal, Thiago, Cátia, Renato, Patrick e todas as outras pessoas que treinam diariamente comigo e fazem parte do meu cotidiano. Obrigada pela amizade, constante motivação e por me fazerem acreditar que eu sou mais forte do que eu imaginava em todos os níveis da minha vida.

A minha querida e amada familia: Antônio (*in memorium*), Terezinha (*in memorium*), Luís, Ricardo, Thais, Sérgio, Thatiane, Roberto, Isabel, Karina, Sophia, Gabriel, Beatriz, Lucas. Mesmo longe vocês foram inestimáveis para mais esta conquista.

Aos meus irmãos, Rainer e Fabio, obrigado pela imensurável ajuda. Obrigado pela ajuda financeira, pelo apoio, amor, visitas (O México sem vocês não seria a mesma coisa), não existem palavras para expressar o quanto vocês fazem parte dessa conquista!! Amo vocês!!!!

Aos meus pais, Angela Lugo e Rainer Ladewig (*in memorium*), não existem palavras para expressar a minha gratidão, amor e carinho para tudo o que vocês fizeram por mim. Obrigado por acreditarem nos meus sonhos e me proporcionarem uma carreira (sem vocês eu não sei onde estaria). Amo tudo o que vocês fizeram para me trazer até aqui. Vocês são o meu orgulho, porto seguro e inspiração para viver, amo vocês com todo o meu ser!!!!!

Palavras-chave

Transplantes, biomonitoramento *in situ*, ecotoxicologia, biomarcadores, estress oxidativo, bivalves, *Mytilus galloprovincialis*, *Rangia cuneata*, *Corbicula fluminea*, *Crassostrea virginica*, sistemas aquáticos, pesticidas, contaminantes

Resumo

Nas últimas décadas a ecotoxicologia teve um enfoque maior nas zonas temperadas, mas com o recente crescimento econômico dos países tropicais, existe uma necessidade de proteger e entender os efeitos deleterios causado por contaminantes em organismos nesses ecossistemas. Com essa necessidade emergente, adquirir dados e entender melhor os efeitos de poluentes em organismos aquaticos tropicais tornou-se crucial, especialmente para relacionar esses efeitos locais ao de larga escala. Atualmente estudos que englobam tais processos estão em falta, o que torna este projecto muito importante para melhor interpretar as respostas biológicas de bivalves e dessa forma melhorar os processos de avaliação de risco relacionados a estes organismos.

De forma a comparar respostas de organismos de zonas tropicais e temperadas, dois experimentos de transplante foram realizados no México e em Portugal (um em água doce e outro em estuário). As respostas a esses biomonitoramentos *in situ* foram obtidas atráves das atividades enzimáticas de diversos biomarcadores como a acetilcolinesterase (AChe), glutationa S-tranferase (GST), catalase (CAT), glutationa redutase (GR), superoxido dismutase (SOD) e peroxidação lipidica (LPO). Estas ferramentas foram usadas para comparar as respostas dos organismos em diferentes ecosistemas e quando comparamos o experimento como um todo existe uma clara diferença entre a resposta de organismos de água doce e os estuarinos. Entretanto, a resposta dos organismos mostra algumas similaridades quando comparamos os resultados das Análises de Componentes Principais (PCA), onde os valores iniciais são diferentes dos outros pontos e os tempos amostrais trazem mais similaridades entre pontos e por tempo depois do segundo tempo amostral.

No laboratório, os ensaios ecotoxicológicos foram realizados com endosulfan e um poluente organico persistente (amostra de um derrame de petroleo ou benzo(a)pireno). O efeito desses compostos foi testado em um organismo padrão (por exemplo: *Mytilus galloprovincialis*) com o intuito de obter uma resposta mais específica, utilizando os mesmos biomarcadores do experimento de campo, com dois dos principais contaminantes em ambientes tropicais. Os organismos escolhidos foram *Rangia cuneata* no México e *Mytilus galloprovincialis* em Portugal. Em geral os organismos foram mais afetados pela amostra de petroleo ou pelo benzo(a)pireno do que pelo pesticida. Hidrocarbonos policiclicos aromaticos (PAH) ou compostos derivados do petroleo tendem a ser mais tóxicos do que pesticidas. Observando os PCAs para os bioensaios com bivalves é claro que após um certo periodo, não existem muitas diferenças entre pontos, especialmente depois de um periodo superior a dez dias.

Keywords

Transplants, *in situ* biomonitoring, ecotoxicology, biomarkers, stress oxidative, bivalves, *Mytilus galloprovincialis*, *Rangia cuneata*, *Corbicula fluminea*, *Crassostrea virginica*, aquatic ecosystems, pesticides, contaminants

Abstract

In the last decades ecotoxicology has focused in temperate zones, but with tropical countries having more economic power, there is a need to protect and understand the deleterious effects of contaminants to organisms in these ecosystems. With this emerging need, it is very important to acquire data and to better understand the effects of pollutants in tropical aquatic organisms and in that way related to a large-scale effects of pollution and other stressors like habitat change on the health status of different bivalve species. Nowadays studies that encompass such processes are lacking what makes this project very important to better interpret the biological responses of bivalves and that way improve the risk assessment processes related to this organisms.

In order to compare responses of organisms from tropical and temperate zones, two transplant experiments were realized in Mexico and in Portugal (one in freshwater and another in estuary). The responses for this were obtained through the enzymatic activity of several biomarkers like acetilcholinesterase (AChe), glutathione S-transferase (GST), catalase (CAT), glutathione reductase (GR), superoxide dismutase (SOD) and lipid peroxidation (LPO). These tools were used to compare the responses of organisms in different scenarios and there is a clear difference between responses from freshwater to estuarine organisms when comparing the whole experiments. Nevertheless, they show some similarities when comparing the results from the Principal Component Analysis (PCA), where initial values differ from other points and sample times and more similarities appears between points and times after the second sample period.

In the laboratory, ecotoxicological assays were performed with endosulfan and a persistent organic pollutant (oil spill sample or benzo(a)pyrene). The effects of these compounds were tested in a model species (i.e. *Mytilus galloprovincialis*) with the aim to obtain a more specific response of the organism, in the same endpoints used in the field experiments, when exposed to two of the main tropical contaminants. The organisms selected were *Rangia cuneata* in Mexico and *Mytilus galloprovincialis* in Portugal. In general the organisms were more affected by the oil spill sample or the benzo(a)pyrene than the pesticide. Normally polycyclic aromatic hydrocarbons (PAH) or compost derived from petroleum are more toxic to aquatic organisms than pesticides. Observing the PCA for the bioassays with bivalves it is clear that after an certain period, there is not so much differences between points, especially after a period superior of 10 days.

INDEX

List of Tables

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

principal component axis. Principal component loading and total variance associated with each axis are provided in Table 6.

Figure 13 – Plot of variable vectors for the two dominant components produced by biomarkers (AChe, BChe, PrChe, GST, CAT, GR, LPO) and C.I. of an exposure with endosulfan.

Figure 14 – The distribution diagram of the different groups of endosulfan concentrations during different experimental periods as a function of the two principal component axis. Principal component loading and total variance associated with each axis are provided in Table 7.

CHAPTER

1

GENERAL INTRODUCTION

1. General Introduction

The human species alters deeply the environment in which is inserted. With the industrial development, the contamination of water, air and soil became a global problem. Indeed, the fast growth of both the industrial activity and the population in the last century resulted in a rapid increase in the degradation of the aquatic environments. Freshwater, coastal and estuarine biota suffers with the rising inputs of organic and inorganic chemicals like hydrocarbons, pesticides, and metals among others. Some of these environmental contaminants may be present at toxic levels, inducing adverse biological (Widdows and Donkin, 1991). However, in the end of the XX century, the society began to be awareness of the importance of environment preservation, leading to a remediation of the Industrial Revolution consequences. These consequences, coupled with the lack of ecological responsibility of mankind in relation to the surrounding environment, are primarily responsible for the environmental damages.

The idea of an environmental monitoring program begin to make sense in the early 1970's when the decision makers started to realize that pollution is an actual and relevant problem. Several biological-monitoring programs started in this period, like the NOAA "Mussel Watch" (1976) and the French "RNO/ROCCH (National observatory network on marine environment quality)" (1974). Both these programs were associated with the assessment of marine ecosystems. Continental water systems (as streams and rivers) have a higher complexity (variety of hydrosystems, large number of aquatic biota and higher number of sampling stations), making it more difficult to maintain a program. It was only in the 90s that biomonitoring programs of rivers became more common; like the American "NAWQA (National Water-Quality Assessment)" (1993), the Belgium "Flemish Eel Pollutant Monitoring Network" (1994) and the French "Plan National PCB" (2008) (Besse et al., 2012).

Environmental monitoring programs are performed in the preparation of environmental assessments, as well as in circumstances in which human activities pose a risk to the natural environment. Aquatic monitoring can include sediment, water sampling or biota (also called biomonitor species) (Kördel et al., 2013). Biomonitors are organisms used to establish

geographical and/or temporal variations in the bioavailability of contaminants, based on causal relationships between contaminants and observable biological effects in the organisms. The use of biomonitors has many advantages: 1- the concentrations found in biomonitors tell more about the bioavailability of the pollutant in the environment than sediment-water samples, which might show large temporal and seasonal variation; 2- biomonitors have wide geographical distribution and contamination levels can be compared internationally (Rainbow, 1995; Zhou et al., 2008). Including biomonitors in the monitoring programs also represents an economical advantage since, for instance, several studies demonstrated that time-scale monitoring based only in water-sediment samples might be difficult and expensive (Phillips, 1977). In addition, inventory-based chemical monitoring of impacted environments is restricted to the identification of a limited number of substances without providing information on their biological significance (Livingstone and Pipe, 1992).

According to Moraes (2001) the interactions that occur between contaminants and a biological population could induce a sequence of structural and functional changes in the higher levels of organization. Subsequently, each physiological effect can cause harmful disturbances of vital functions integrated, which can affect the population. So the existence of a population is a function of all the responses of individuals to environmental changes.

A good biomonitor should be abundant in the area, have long life span for comparison between various ages, sufficient sampling size and hardiness to tolerate laboratory incubation. The species should be relatively easy to identify and to sample. Additionally, the species has to tolerate and accumulate contaminants without suffering mortality and a well dose-effect relationship can be observed. To reflect environmental status in a specific area, the species should be sessile or have slow or limited range of movements (Zhou et al., 2008).

Bivalve molluscs, such as mussels and oysters, have been widely used to assess stress and chemical contamination in the aquatic environment (Goldberg et al., 1980; Rainbow, 1995; Tomazelli et al., 2003; Sarkar et al., 2008; Bérges-Tiznado et al., 2013; Lacroix et al., 2015). Despite

bioaccumulation of toxic chemicals in their tissues the interaction and potential effects of these compounds on living organisms and on their environment are not easily reflected (van der Oost et al., 1997). In particular, they are mainly used for monitoring spatial and temporal changes in chemical contamination of the aquatic environment. This is due to their ability to pump large volumes of water, bio-concentrate metals and organic contaminants in their tissues and limited ability to metabolize such contaminants (Casas and Bacher, 2006; Hamza-Chaffai, 2014; Kim et al., 2008; Rocher et al., 2006).

1.1 The use of bivalves as biomonitors

Mussels (**Fig. 1A**) are the most common group used as biomonitors in marine monitoring programs (Escartín and Porte, 1997; Gorbi et al., 2008; Lacroix et al., 2014; Ritz et al., 1982; Spada et al., 2013). Mussels are especially useful as biomonitors when there are multiple or unknown pollutants and the net effect on the mussels may be additive. Among the most common mussel species (or genus) used as biomonitors there are *Mytilus edulis*, *Mytilus galloprovincialis* and *Mytilus trossulus*.

Oyster (**Fig. 1B**) represents the second most common group of bivalve molluscs employed as biomonitors. Like mussels they are widespread suspension feeders in coastal waters and relatively easy to identify. The main species used for monitoring are from the genus *Crassostrea* (i.e. *C. gigas, C. virginica, C. margaritacea* and *C. brasiliana, C. angulata)* and have been widely spread through the world for mariculture purposes. Despite both oysters and mussels are highly recommended for monitoring programs, oysters have been less studied than mussels, thus having a less extensive literature in the biomonitoring studies (Griffith et al., 2013; Lauenstein et al., 2002; Luna-Acosta et al., 2010; Wanick et al., 2012; Wirth et al., 1996).

Some bivalves have not been used as often as biomonitors, as is the case of the brackish water clam *Rangia cuneata* (**Fig. 1C**). This species is considered native to the Gulf of Mexico and introduced to the NW Atlantic, where it is predominantly found in estuaries (Verween et al., 2006). According to S. H. Hopkins & Andrews (1970), there were no sightings along the U.S. Atlantic coast until 1956, where the species was thought to be extinct since the Pleistocene. The presence of *R. cuneata* in Europe was first recorded by

Verween et al. (2006) in the harbor of Antwerp, Belgium and; started to spread rapidly over other regions of Europe, as Vistula Lagoon of the Baltic Sea (Ardura et al., 2015; Rudinskaya and Gusev, 2012; Solovjeva, 2014). The appearance and the increase of abundance in NW Atlantic and Europe could be due to the transportation as larvae and/or juveniles in ballast water (Pfitzenmeyer and Drobeck, 1964; Verween et al., 2006; Rudinskaya and Gusev, 2012). This could lead to a massive case of invasive species and, according to Ardura et al. (2015) *R. cuneata* is a good candidate for the World's "black list" of invaders, highlighting the need to study this species.

When the subject is non-indigenous invasive species, *Corbicula fluminea* (**Fig. 1D**) is recognized as one of the most important in the aquatic ecosystems (McMahon, 2002; Sousa & Rufino, 2008; Rosa et al., 2014; Gama et al., 2016). In the last 80 years, species of the *Corbicula* genus have extended its range from Asia, Oceania and Africa to American and European ecosystems (McMahon, 2002; Mouthon, 2001a, 2001b; Bódis et al., 2012; Früh et al., 2012; Lucy et al., 2012; Kamburska et al., 2013). Special consideration should be given to the invasive specie *C. fluminea* that carry new attributes to ecosystems and species that dominate communities as virtual monocultures and thereby have the potential to disrupt ecosystem processes (Hall et al., 2006). The wide distribution of *C. fluminea* offers the opportunity for an intercontinental in situ biomonitoring research using a single test organism (La Guardia et al., 2012).

The ability of bivalves to alter some characteristics (metabolic rate, biomass, etc.) and to concentrate pollutants in their bodies may be affected by some environmental factors. These factors include temperature, salinity, season and organic matter (Vercauteren and Blust, 1996; Hagger et al., 2010), body size (Wang & Fisher, 1997; Sokolowski et al., 2004; Mubiana et al., 2006; Zhong et al., 2013), sex and reproductive status (Widdows & Donkin, 1992; Richir and Gobert, 2014; Liu et al., 2014), tidal height (Lobel and Wright, 1982) and physiological condition (Marsden et al., 2014; Nilin et al., 2012; Widdows and Donkin, 1991). However, effects of these factors in quantitative terms still remain unclear with different results reported depending on the study, element, location or season (Rainbow, 2002).

Figure 1. Species used in transplants in Mexico and Portugal. (A) *Mytilus galloprovincialis* (B) *Crassostrea virginica* (C) *Rangia cuneata* (D) *Corbicula fluminea*.
(Source: (A) http://www.discoverlife.org/mp/20q?search=Mytilus+galloprovincialis; (B) http://www.sealifebase.org/summary/Crassostrea-virginica.html; (C)http://www.elrincondelmalacologo.com/Web%20fotos%20marinos%20no%20gasteropodos/Mactridae.htm; (D) http://lhprism.org/species/corbicula-fluminea)

In the original context biomarker was defined as a change in a biological response (ranging from molecular through cellular and physiological responses to behavioral changes), which can be related to exposure to or toxic effects of environmental chemicals (Peakall, 1994). Biomarkers measure changes at the biochemical, cellular and physiological levels in order to assess the health of aquatic organisms related to the quality of their environment. Biomarkers have been used as effective early warning tools in ecological risk assessment and aquatic environment monitoring (Cajaraville et al., 2000). Biomarkers based on responses at molecular and cellular level represent the earliest signals of environmental disturbance and are commonly used for monitoring purposes. Among these, some of the most commonly used types of biomarkers are those linked to metabolism and energetics,

because this permits the biochemical response to pollutants to be related to changes at higher levels of biological organization (Depledge et al., 1995). Others are those related to physiological responses (inhibition of cholinesterases (ChE), induction of glutathione S-transferases (GST), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and levels of lipid peroxidation (LPO)), which will offer a very high level of sensitivity, making it possible to detect a low-level cellular response to organic or inorganic pollutants.

Cholinesterases (Ches) are specialized enzymes that play a central role in neurotransmission (Jebali et al., 2013; Kaufer et al., 1998; Soreq and Seidman, 2001; Vale et al., 2003). There are three cholinesterases characterized in bivalves: AChe, BChe and PrChe (Mora et al., 1999; Talesa et al., 2001). The inhibition of acetylcholinesterase (Ache) was previously applied as a specific biomarker in response to organophosphate and carbamate pesticides (Führer et al., 2012; Fulton and Key, 2001; Gagné et al., 2010; Viarengo et al., 2007). For several years, BChe and PrChe were included in the generic group of pseudocholinesterase, but they have not been distinguished from one another yet (Gambardella et al., 2013). The authors Pezzementi & Chatonnet (2010) proposed, after analysis of molecular forms, that both BChe and PrChe are post-transcriptional precursors of Ache.

Contaminants when entered the organism system undergo an oxidative process of biotransformation to become less toxic and be excreted easier through detoxification mechanism. The metabolites or by-products of previous stage (Phase I) do the most serious damage to organism, which need other enzyme to be metabolized further. GST activity is part of the phase II of the metabolism process and is responsible to the conjugation of electrophilic compounds; the toxicity of several compounds can be modulated by its induction (van der Oost et al., 2003). Another detoxification pathway is related to antioxidant enzymes and enzymes like SOD, CAT and GR attack and eliminate reactive oxygen species (ROS), which are generated during the contaminants detoxification-related process and could be more dangerous to organisms than the pollutant itself (Livingstone et al., 1992). Failure of antioxidant defenses can lead to oxidative damage including enzyme inactivation, protein degradation, DNA damage and, lipid peroxidation (LPO)

(Altenburger et al., 2003; Halliwell and Gutteridge, 1999; Regoli and Giuliani, 2014).

1.2 The use of bivalve transplants in biomonitoring

Nowadays, new trends in monitoring are emerging and active programs, where individuals are transplanted or caged from reference sites, are becoming more reliable and common. According to a review of Besse et al. (2012) there are several advantages in the use of active monitoring programs, some of them include the choices of sites and consequently the known exposure duration, the possibility to repeat the experiment summed with the predictability of costs and duration. But, the most important benefit is the choice of stock leading to a control of biotic parameters (such as genetic or age variability, phase of reproductive cycle and metabolic activity), which may have strong influences when comparing data from different wild populations.

The utility of transplanted bivalves has been proven to be a useful strategy in monitoring programs (Gorbi et al., 2008; Gunther et al., 1999; Hédouin et al., 2011a; Nasci et al., 2002; Nigro et al., 2006; Phillips and Segar, 1986; Regoli and Orlando, 1994, 1993; Regoli et al., 2004). By using these organisms, it is possible to measure the actual bioavailability of contaminants in the investigated area, reducing the effects of the biological variables allowing us to fill gaps in our knowledge on mechanisms of effects of persistent organic pollutants in wildlife. According to Gorbi et al. (2008) caged bivalves facilitate the investigation in areas where native organisms are absent; reducing the influence of seasonal variability or adaptive phenomena, factors that can induce bias in data and undermine the capability to discriminate among different levels of environmental disruption.

Monitoring programs can be carried out with several species or with a single species (single species in situ assays), the use of transplanted organisms offer a more ecologically-realistic approach over controlled conditions in the laboratory or over the use of resident species (Crane et al., 1996; Hédouin et al., 2011b, 2008). Whole-organism endpoints such as death, morphological deformities and growth, provide general measures of stress and can be used to detect changes in environmental quality. Molecular-

level responses may be more toxicant specific and have the potential to be used as diagnostic tools. In this case, caged bivalves are normally used for the analysis of chemicals accumulation and later are linked to the physiology responses of the organisms through biomarker analysis. For example, inhibition of Ache has been used as an indicator of organophosphate and carbamate pesticide exposure, and inhibition of aminolevulinic acid dehydratase has been used as an indicator of lead exposure (Giarratano et al., 2010; Gorbi et al., 2008; Ng et al., 2013; Nigro et al., 2006; Peakall, 1992; Roméo et al., 2003).

1.3 Organic contaminants and his impact in the environments

The increased use and production (sometimes as by-products) of compounds like hydrocarbons and pesticides over the past half century reflects the nature of life in the developed world and increased industrialization. This has resulted in organic compounds, particularly those resistant to degradation (often referred to as persistent organic pollutants or POPs) becoming ubiquitous in the environment, with a truly global distribution (Warren et al., 2003).

Petroleum products are a widespread class of environmental contaminants that may enter the aquatic environment through discharges of industrial and urban effluents, shipping activities, offshore oil production, oil spills, fossil fuel combustion, and natural seeps (Medeiros et al., 2005). These products consist mainly of saturated noncyclic hydrocarbons, cyclic hydrocarbons, oleofinic hydrocarbons, aromatic hydrocarbons, sulphur compounds, nitrogen–oxygen compounds and heavy metals. However, each crude oil or refined product widely varies in its chemical composition and physical properties depending of its origin (Wake, 2005). Following entry into the aquatic environment, these contaminants may suffer physical, chemical and biological alterations through weathering processes, which can be considered as one of the main factors influencing the toxicity and the potential ecotoxicological effects of these environmental contaminants (Neff et al., 2000).

Among the variety of natural and anthropogenic contaminants in the aquatic environment there are many that are genotoxic, i.e. capable of

interacting with the genetic material. For example, polycyclic aromatic hydrocarbons (PAHs) have well documented toxic, mutagenic and carcinogenic properties, which along with the fact that they are widely distributed in the environment, makes them priority pollutants (Neff, 1979; Grimalt et al., 2004; Guo et al., 2007; Cao et al., 2010; Johnston et al., 2015).

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental persistent organic pollutants and have mainly two major sources: anthropogenic and natural. Anthropogenic sources include discharges from electrochemical industry such as aluminum smelters (Lorenz and Glovik, 1972; Neff, 1979), incomplete combustion of coal, oil, fossil fuel and biomass (Jiang et al., 2011; Qin et al., 2013; Rogge et al., 1993; Simoneit, 1984; Sun et al., 2016; Tao et al., 2005; Xu et al., 2006), spills and leakages from the extraction of crude oil and its products (Dudhagara et al., 2016; Witt, 1995; Wolska et al., 2012; Xu et al., 2007). Natural sources consist of forest fires, volcanic eruptions, natural leakage, diagenesis of organic matter and synthesis by plants (Wolska et al., 2012).

PAHs can enter the aquatic environment through wastewater discharge, urban surface runoff, atmospheric precipitation and ship recycling activities (Dudhagara et al., 2016; Durand et al., 2004; Heemken et al., 2000; Nadal et al., 2004; Qin et al., 2013; Rahmanpoor et al., 2014) leading to a potential hazardous impact on organisms from this ecosystem. Chronic exposure to PAHs poses a threat to aquatic biota and wildlife (Peterson et al., 2003). This is of special concern in areas where there is generally chronic and uncontrolled PAH contamination from multiple sources such as highly urbanized estuaries.

Compounds as PAHs have usually a permanent effect on aquatic organisms due to the their hydrophobicity, the compound can be easily taken up by marine organisms because of their ability to interact with cellular molecules following binding to lipophilic sites. If the target is a key molecule of a cellular process, a toxic response may be induced, and, at the extreme, the integrity of the organism can be seriously compromised (Chen et al., 2013; Johnston et al., 2015; Meador, 2003). The lipophilic character of PAHs indicate the tendency to enter the food chain and the food web, magnified to several trophic levels can affect eventually the human health (Qin et al., 2013;

Wang et al., 2010), hence the importance to study the fate in all compartments that can cause adverse effects on the human life.

Benzo[a]pyrene (B[a]P) belongs to the PAH group and its major source is from man-made activities involving the combustion of coal, oil, wood, diesel and petrol (Maria and Bebianno, 2011). This xenobiotic is included in the list of priority pollutants of the US Environmental Protection Agency (US EPA) due to its toxicological features (EPA, 1995); it is a common contaminant of estuaries, coastal areas and other aquatic ecosystems. Sublethal amounts of B[a]P are commonly found in aquatic environments specially after oil spills accidents (Banni et al., 2010; Volodkovich and Belyaeva, 1992).

Benzo[a]pyrene (B[a]P) is one of the most extensively studied genotoxicants in aquatic organisms (Ching et al., 2001; Clements et al., 1994; Kim et al., 2014; Lyons et al., 2002; Stein et al., 1984; Varanasi and Gmur, 1981; Wu et al., 2016). The bioaccumulation mechanisms of B[a]P and other carcinogenic PAHs in marine organisms is of interest to aquaculture, and in the utilization of these marine resources for human food, thus making it essential to understand the fate of such organic contaminants within these organisms (Moore et al., 1986).

Among pollutants, pesticides have become more common in estuarine areas. They are mainly introduced into rivers via run-off and then may enter marine areas, particularly estuarine and coastal zones. These pollutants may have major ecological consequences and could endanger organismal growth, reproduction or survival (Banerjee et al., 1996).

A pesticide is defined as a chemical substance used to control, repel, attract, or kill pests, for example, insects, weeds, birds, mammals, fish, or microbes that considered to be a nuisance (Rathore and Nollet, 2012). The type of organism often classifies pesticides: fungicides, herbicides, insecticides, nematocides and rodenticides (Renault, 2011). After the Second World War the concern with production of food for a growing world population and the need for pest control agents, established the indiscriminate use of DDT (dichlorodiphenyltri-chloroethane) and the some other persistent pesticides; all banned nowadays. The impact of this use to man and environment's health was pronounced and after the release of Rachel

Carson's book Silent Spring (1962) there was embedded a feeling of concern and the need to understand the impacts and the mode of action of pesticides (Stenersen, 2004).

The use of pesticides in the actual days is a problem not only related to the degradation of the environment, but it can be linked to a health issue. There are several articles (Bolognesi, 2003; Konradsen et al., 2003; Eddleston et al., 2008; Buckley et al., 2011; Hernandez et al., 2013) concerned to the acute poisoning of humans mostly in the developing countries. The monitoring of pesticides levels in organisms and/or sediments could lead to a health status of the habitat and his fauna. Since bivalves are an important food source to humans, it is very important to have studies with these organisms to assess how much they accumulate and how much it can be carried to the next level of the food chain (**Fig. 2**).

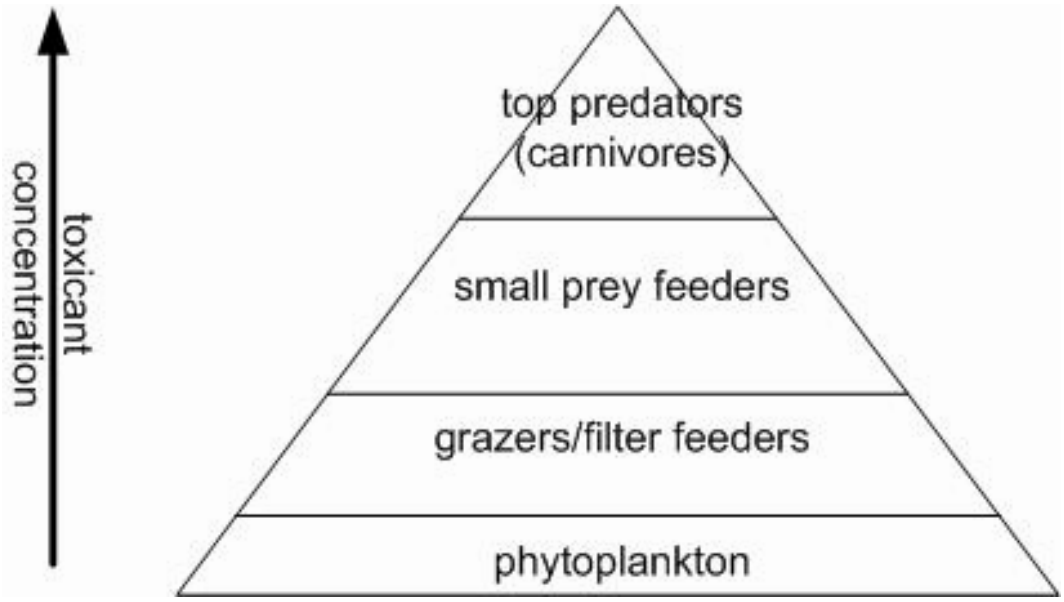

Figure 2. Biomagnification. Increase in toxicant concentration in tissue at higher trophic levels due to dietary uptake versus biomass (Lehmann, 2002).

Endosulfan is an organochlorine insecticide and acaricide that has been one of the most widely used non-systemic pesticide throughout the world since its creation in 1954 (Becker et al., 2011; Miller and Llados, 1999; Shao et al., 2012). Endosulfan is an extreme versatile pesticide and its use has led to extensive worldwide use in the agriculture and in different crops

(including cotton, cereals, fruits, vegetables, tea and coffee) (Becker et al., 2011; Weber et al., 2010). The use of endosulfan has been discontinued or restricted in European and North American countries, however it is still widely employed in developing countries, what have a global impact since its residues can reach various geographical regions, such as the artic and the Himalayans (Becker et al., 2011; Da Cuña et al., 2011; Loewen et al., 2005; Shao et al., 2012; Weber et al., 2010).

Endosulfan can enter aquatic environments following utilization through runoff of contaminated surface soil from rain events and accidental releases or from drift or overspray of aerial applications (Miglioranza et al., 2002; Pablo and Hyne, 2009) and cause adverse effects to non-target aquatic animals. EPA postulates that concentrations of endosulfan above 0.22 μg/L (acute) and 0.056 μg/L (chronic) have and adverse impact on the health of aquatic organisms (Ballesteros et al., 2009; Mersie et al., 2003). The European Commission Directive on the Quality of Water Intended for Human Consumption sets a maximum admissible concentration of 0.1 μg/L per individual pesticide and 0.5 μg/L for the sum of pesticides in drinking water (EU, 1998). The potential human health impacts include cardiovascular, endocrine, gastrointestinal and respiratory toxicity (ATSDR, 2000; Capkin et al., 2006). Endosulfan concentrations in aquatic environments are generally low (<1 μg/L) (Capkin et al., 2006), however in areas of application has been found in surface and groundwater a range from 0.05 to 2.5 μg/L (Da Cuña et al., 2011; Dalvie et al., 2003; Leong et al., 2007).

1.4 Portugal and the Temperate scenarios in Ecotoxicology

The ecotoxicology field has mainly focus in countries and temperate zones, especially because in the North Hemisphere the concern with the impacted areas started around 1970 with the creation of several monitoring programs. Through these monitoring programs and along this past decades several tools were created and developed to better understand the fate, effects on organisms and ecosystems of contaminants. Even with all the researches and programs they are still a lack of data from several places and/or organisms, like the Ria de Aveiro that has several studies regarding

metal contaminants (Abreu et al., 2000; Hall et al., 1987; Martins et al., 2013; Oliveira et al., 2010; Pérez Cid et al., 2001) and even some with hydrocarbons (Cunha et al., 2006; M. Pacheco et al., 2005; Serafim et al., 2013) but has a lack of studies with pesticides (Antunes and Gil, 2004).

Ria de Aveiro, a coastal lagoon located in the northwest of Portugal, has a significant role in the life cycle of several organisms. Over decades, this lagoon has been the main receptor of anthropogenic discharges resulting mainly from chlor-alkali (Pereira et al., 1997) and pulp/paper plants (Pacheco et al., 2005), harbour and dry-dock activities (Barroso et al., 2000; Pacheco et al., 2005), municipal and domestic effluents (M Pacheco et al., 2005) and chemicals used in agriculture (Monteiro et al., 2007). Though in the year 2000, point source discharges were diverted through a submarine outlet 2.5 km far from the seacoast, recent studies have demonstrated the continuation of critical areas (Ahmad et al., 2008; Oliveira et al., 2009; Ramiro Pastorinho et al., 2012; Santos et al., 2004, 2006), revealing the imperative need of an effective biomonitoring program (Oliveira et al., 2010).

This estuarine system has a high economical potential, supporting fishery and aquaculture activities, as well as ports, dockyards and industry facilities. In addition it is classified as a special protected area by the EU nature and biodiversity policy ‘Natura 2000 Network’. This identifies the Ria de Aveiro as an ecosystem of considerable importance, requiring active management of its environmental and ecological quality (Galante-Oliveira et al., 2009).

The River Minho is normally used in ecotoxicological studies as a reference site because of the very low human pressure (Cairrao et al., 2004; Moreira et al., 2006; Quintaneiro et al., 2006; Monteiro et al., 2007; Dagnac et al., 2012; Mil-Homens et al., 2013; Capela et al., 2016). In the course of the river in Portugal, there is a population of approximately 25 000 people, distributed by the councils of Caminha, Vila Nova de Cerveira and Valença, dedicated essentially to the primary sector. There is a low level of industrialization and the main problems of contamination are mainly related to agricultural and industrial pollution sources as well as discharges of untreated domestic effluents or deficient treatment (Santos et al., 2013). In the last years there is an expansion of an important industrial pole in the council of

Vila Nova de Cerveira and constructions of dams, where the discharge could bring some threats (Santos et al., 2013). In the Spanish side, the industrial activity is intense, especially in the manufacturing of Orense and Porriño Industrial Park (Alves, 1996; PBH, 2001).

The estuary of Minho River, located in the border of Portugal and Spain, is considered as one of the least contaminated along the Portuguese coast and was classified as a Natura 2000 site. The Minho River originates in Serra da Meira, in the province of Lugo (Spain) and drains into the Atlantic Ocean. It is >300 km long, the last 70 km of which comprises its international section (Sousa et al., 2005). The variety of habitats available (e.g. saltmarsh, sandflats, mudflats and freshwater tidal habitats) and relatively high species diversity made this estuary an ideal site to investigate the use of molluscs in monitoring programs of freshwater-estuarine transitional ecosystems, since very little attention has been devoted to this issue.

1.5 Mexico and the Tropical scenarios in Ecotoxicology

Ecotoxicology in general has focused almost exclusively on countries and ecosystems in temperate zones, but this is a reality that is slowly changing during the past years. Especially because tropical ecosystems combined contain 75% of the global biodiversity, and the developing countries started to have a real concern with the remediation of the polluted areas. But even with great interest of the scientific community and the increased amount of projects focused in the effects of contaminants in organisms from the tropical zones, it is still a lot of data to generate in the way to have a better regulation of the contaminants in these environments.

Environmental behavior of organic chemicals, as pesticides and hydrocarbons, may be different between temperate and tropical conditions. The basic elements important when comparing contaminants dissipation in these ecosystems are temperature, rainfall, sunlight and microorganisms (Daam and Van den Brink, 2010; Magallona, 1994).

Several studies suggest that degradation rates and toxicity might be higher in tropical countries due especially to higher temperatures (Boone and Bridges, 1999; Brecken-Folse et al., 1994; Buckman et al., 2007; Capkin et al., 2006; Gaunt and Barker, 2000; Howe et al., 1994; Lydy et al., 1999;

Monserrat and Bianchini, 1995; Noyes et al., 2009; Sanchez-Bayo and Hyne, 2011; Sethunathan, 1989; Sprague, 1985; Viswanathan and Murti, 1989). Higher ambient temperatures are responsible for the increase in hydrolysis rates in tropical waters, favoring dissipation of pesticides (Bailey, 2004; Benitez et al., 2006; Chai et al., 2009; Sanchez-Bayo and Hyne, 2011; Sharmila et al., 1988; Van Den Berg et al., 1999) and influencing the environmental fate and behavior of persistent organic pollutants, such as hydrocarbons and PAHs (Brubaker and Hites, 1998; Ma et al., 2004; Macdonald et al., 2002; Meyer and Wania, 2008; Noyes et al., 2009; Sinkkonen and Paasivirta, 2000; Sweetman et al., 2005; Wania, 1999). However, biochemical detoxification and elimination of the chemical may also increase with temperature (Arbeli and Fuentes, 2007; Howe et al., 1994; Racke et al., 1999; Sanchez-Bayo and Hyne, 2011), which may cause a decrease in chemical toxicity, depending on species- specific physiology (Castillo et al., 1997).

Terminos Lagoon is a vast system comprised of rivers, estuaries, a coastal lagoon and deltaic areas. The Lagoon receives freshwater discharges of four main rivers (Usumacinta, Palizada, Chumpan and Candelaria) and communicates to the sea through two inlets (at Paso Real and Ciudad del Carmen), though the magnitude of the riverine-marine influence is still unknown (Carvalho et al., 2009; Magallanes-Ordóñez et al., 2015). Terminos Lagoon is the largest lagoon in the shouthern Gulf of Mexico and one of the most studied costal systems in Mexico due to its biological diversity and its importance as a nursery and feeding area for various fish and shrimp species (García-Ríos et al., 2013; Yañez-Arancibia and Day Jr, 1988). The Lagoon possess a high economic and ecological importance, for this reason the Mexican government declared this region and adjacent a Protected Natural Area in 1994 (Abascal-Monroy et al., 2015; INE/SEMARNAP, 1997).

The ecosystem has been subject to intense multiple pressure due to anthropogenic activity during recent decades (Sirot et al., 2015). These stressors include development of oil industry, wetland loss, mangrove deforestation associated with crop and livestock development, and urban development in the adjacent areas (Abascal-Monroy et al., 2015; Vázquez-Luna, 2012). The agriculture activities mainly rice production, and cattle

feeding in the watershed of main rivers discharging into the Laguna de Terminos, may originate the release of organic pollutants, which ultimately disseminate in the lagoon. Pesticides used in rice fields include chlorpyrifos, carbofuran, molinate and glyphosate as well as endosulfan, parathion, malathion, methomyl, benomyl, and dichlorophenoacetic acid (Rendón von Osten et al., 2004; Rendón-Von Osten et al., 2006). Since organic contaminants have a tendency to affect the aquatic biota it is very important to maintain a monitoring program, especially because the grown of human settlements in the region and subsequent development of economic activities have put an increased ecological stress on the ecosystem (Díaz-de-León et al., 2004).

Others ecosystems from Mexico are not so intensely studied and monitored, one example is the Champoton River, however this is changing from the past years and several studies have been conducted (López-López et al., 2009; Rendón et al., 2008; Trujillo-Jiménez & Sedeño-Díaz, 2011; Trujillo-Jiménez et al., 2014). The Champoton River is located in the humid tropics of Southeastern Mexico, in terrain with a high content of karstic material, and is the main surface stream in the Yucatán Peninsula (López-López et al., 2009). This river is within the so- called hotspot of Mesoamerica (Myers et al., 2000), whose main problems are agricultural waste input, discharges from a sugar mill and contamination by domestic sewage at the mouth of the river. Río Champotón is particularly relevant due to attributes associated with ecosystems amenable to conservation, although it faces major challenges from deforestation and non-point source pollution (López-López et al. 2009; Trujillo-Jiménez et al. 2014).

1.6 Objectives and outline

The main aim of this study is to assess the effects of different pollutants (PAHs and pesticides) on physiological parameters of bivalve species from two locations (Mexico and Portugal) of the Northern Hemisphere. The tested hypotheses are: (1) Different bivalve species (*Mytillus galloprovincialis, Crassotrea virginica, Rangia cuneata* and *Corbicula fluminea*) have physiological differences in different ecosystems (estuarine and freshwater); (2) The bivalve species used (*Mytillus galloprovincialis,*

Crassotrea virginica, Rangia cuneata and *Corbicula fluminea*) have adverse effects when exposed to pollutants (PAHs and pesticides); (3) Different bivalve species collected from different ecosystems have similar overall responses when exposed to pollutants under related circumstances.

In particular, the objectives of this study are:

(i) Assess the physiological differences of bivalve species in different estuarine systems in the North Hemisphere and to predict the degree of pollution in the different habitats by measuring the physiological stress responses (biomarkers, condition index);
(ii) Assess the physiological differences of bivalve species in different river systems in the North Hemisphere and to predict the degree of pollution in the different habitats by measuring the physiological stress responses (biomarkers, condition index);
(iii) Assess the acute and chronic effects of a specific PAH (benzopyrene), and pesticide (endosulfan) on the physiology of the model bivalve species (*Mytilus galloprovincialis*);
(iv) Determine if different bivalve species sampled from different ecosystems have similar overall responses when exposed to different pollutants (PAHs and pesticides) *in situ*.

This study is an attempt to better understand the large-scale effects of pollution and other stressors like habitat change on the health status of different bivalve species. Currently, studies that encompass such processes are scarce, highlighting the relevance of this project in the interpretation of the biological responses of bivalves and, thus, improving the risk assessment processes related to these organisms.

This thesis is organized in six chapters, including the current one that refers to the Introduction and contextualization of the work here developed (Chapter 1), four chapters in the format of scientific publications and a final chapter of General Discussion and Final Remarks aimed to integrate all the data and highlight major and final conclusions of the present work.

Each chapter can be summarized as follows:

Chapter 1: General Introduction. In this chapter a brief contextualization of the thesis thematic is provided. Namely it intends to identify the knowledge gaps existing in the literature and the aims of the present work. In addition, the outline of the thesis is also described.

Chapter 2: Stress related biomarkers from transplanted bivalves (*Crassotrea virginica* and *Rangia cuneata*) in a tropical ecosystem. In this field study we assessed the effects of *in situ* contamination (in locals of known impact) in the sublethal responses of bivalves (through biomarkers and condition index) over a 4-month period. This study was conducted in two different tropical environments (estuary and river) in Mexico.

Chapter 3. Biochemical responses of a non-indigenous clam (*Corbicula fluminea*) and mussel (*Mytilus galloprovincialis*) to a transplant experiment in different scenarios in Portugal. This chapter describes another field study conducted to assess the sublethal effects of different contaminants to bivalves in two different temperate environments, Minho River and Aveiro Estuary in Portugal.

Chapter 4. Biomarkers as a sublethal tool for short and long-term exposure of benzo[a]pyrene in mussels (*Mytilus galloprovincialis*). In order to understand the field experiments and the physiological behavior of organisms, a model monitor (the mussel *M. galloprovincialis*) was selected to assess the sublethal effects of a PAH (benzo[a]pyrene) in the selected endpoints (biomarkers and condition index) over a short and long-term exposure.

Chapter 5: Effects of endosulfan on neurotoxic and antioxidant enzymes in mussels (*Mytilus galloprovincialis*). To achieve a better link between all data and to facilitate the knowledge of the physiological behavior, short and long-term experiments were performed to assess the sublethal response of the mussel *M. galloprovincialis* trough selected endpoints (biomarkers and condition index).

Chapter 6: General Discussion and Final Remarks. In this chapter we intended to compare and integrate the data obtained in each chapter to reach the proposed objectives, creating an overview of the whole work.

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CHAPTER

2

STRESS RELATED BIOMARKERS FROM TRANSPLANTED BIVALVES (*CRASSOSTREA VIRGINICA* AND *RANGIA CUNEATA*) IN TROPICAL ECOSYSTEMS

Stress-related biomarkers from transplanted bivalves (*Crassostrea virginica* and *Rangia cuneata*) in tropical ecosystems.

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This chapter was submitted as an original article to:

Environmental Monitoring and Assessment

Abstract

Biomarkers are used as tools to assess the effects of environmental pollution. In this study, we aimed to investigate different types of contaminants (locals of known impact) in the sublethal responses of two bivalve species during a transplantation experiment: *Crassostrea virginica* in Términos Lagoon and *Rangia cuneata* in Champoton River. To determine the stress response, several endpoints were chosen as body condition index (CI) and enzymatic activities of acetilcholinesterase (AChe), glutathione S-tranferase (GST), catalase (CAT) and superoxide dismutase (SOD) in the digestive gland of selected organisms. Noticeable effects occurred in the AChe and GST responses for *C. virginica*, suggesting a relation between the location of the points and different sources of pollution. In the experiments of Champoton River exists a clear difference among seasons: higher impact in the endpoints from the rainy season, especially in GST. SOD and CAT responses were less affected in both scenarios; this trend can be linked to a good oxidative system defense of the organisms. Principal Components Analysis (PCA) shows a time-related response of organisms in Terminos and Champoton at rainy season. The opposite situation occurred in the dry season in Champoton River. A higher battery of biomarkers and other physiological endpoints could better elucidate the relationships between organisms and the sources of contaminants.

Keywords: *Crassostrea virginica*, *Rangia cuneata*, transplantation, biomarkers, Términos Lagoon, Champoton River.

1. Introduction

Coastal and estuarine environments are subject of several forms of disturbance, among which chemical pollution linked to industrial production and high urbanization. The fast growth of the industrial activity during the last decades resulted in a rapid increase of the inputs of organic and inorganic chemicals (hydrocarbons and pesticides). Some of these contaminants can induce adverse biological effects, creating the need of environmental monitoring.

Bivalves have been widely used to assess spatial and/or temporal stress of chemical contamination in the aquatic environment. Along the past decades, several authors (Anderlini et al., 1981; Campillo et al., 2013; Cardoso et al., 2015; Goldberg et al., 1978; Oros and Ross, 2005; Regoli and Orlando, 1994) pointed out the use of transplanted bivalves (e.g. mussels, oysters and clams) to monitor aquatic ecosystems and the following effects of pollutants.

The use of *in situ* assays combining general stress and toxicant-specific molecular responses can provide ecologically relevant, sensitive, and diagnostic monitoring tools. A deeper knowledge of toxicity mechanisms at the long-term and the molecular level is required to choose an effective way to deal with the respective pollutant in a risk assessment perspective (Vasseur and Cossu-Leguille, 2006). A multidisciplinary approach would be necessary to validate relevant biomarkers and, the enzymatic response of organisms can be used as effective early warning tools.

Molecular-level responses may be more toxicant specific; for instance, the inhibition of acetylcholinesterase (AChe), widely used as neurotoxicity biomarker, is especially sensitive to carbamate or organophosphorus pesticides (Michel et al., 1998). AChe is an essential enzyme and its main physiological function is the hydrolysis of acetylcholine into choline and acetic acid, the inhibition of this endpoint could lead to severe physiological impairment in the organism, since there is a break in the transmission of nerve impulses across the synapse (Vidal-Liñán et al., 2015).

Glutathione S-Tranferase (GST), phase II detoxification enzyme, is responsible for the detoxification of xenobiotics. Thus, GST is densely used in ecotoxicological-based studies: in bivalves as an endpoint of exposure to

pesticides (Tao et al., 2013), metals and polycyclic aromatic hydrocarbons (PAHs) (Zhang et al., 2012), since it plays an important role in the defense against oxidative damage.

Oxygen toxicity, also known as oxidative stress, might be an effect of exposure to different types of pollutants, namely PAHs (Martins et al., 2013; Sureda et al., 2013), metals (Banni et al., 2014; Di Salvatore et al., 2013; Sabatini et al., 2011), pesticides (Patetsini et al., 2013; Sellami et al., 2014) and pharmaceutical drugs (Parolini et al., 2015; Pedriali et al., 2013). The defense system of bivalves tends to inhibit oxyradical formation, whereas enzymes as superoxide dismutase (SOD), catalase (CAT) and others act on the detoxification of free oxyradicals (O_2^-, H_2O_2, HO^-). Oxidative stress could lead to enzyme inactivation, lipid peroxidation, DNA damage and, consequently cell death (Van der Oost et al., 2003).

Although the use of biomarkers in transplanted bivalves is recurrent, this sort of monitoring conducted in tropical regions is required to be spatial widespread. Tropical ecosystems possess specific characteristics and the organisms will respond differently to the pollutants. The limited number of studies with mollusks (Flores-Nunes et al., 2014; Maranho et al., 2012; Seabra Pereira et al., 2014) supports the paramount need to overlap the gap between temperate and tropical-related knowledge.

In this study we aimed to compare the influence of different types of contamination (rivers inflow-related pesticides and- anthropogenic impact-related hydrocarbons) in locations of known impact in the sublethal response of bivalves during 4 months. We also aimed to assess if the response is similar in different environments: estuary (Términos Lagoon) and river (Champoton River). We hoped to improve the knowledge of *in situ* experiments in tropical environments and the physiological responses of bivalves to environmental contamination in this region.

2.Materials and Methods

2.1 Study sites

2.1.1 Términos Lagoon

Términos Lagoon (Campeche, Yucatan Peninsula, México; **Fig. 1**) has the following dimensions: 70 km (length), 30 km (width), 3.5 m (depth) and a 170,000 ha (surface area). Two permanent inlets connect the lagoon with the Gulf of Mexico: Puerto Real and Carmen (Yañez-Arancibia and Day Jr, 1988). Most freshwater input comes from the Palizada River, which drains the largest basin in Mexico (Contreras, 1993; Yañez-Arancibia and Day Jr, 1988). Average annual precipitation (1,680 mm yr^{-1}) is seasonal, with a June-October rainy season associated with frequent tropical convectional rains. The winter storm, or “Norte” season, goes from November to February, with strong north winds and frontal rains. The dry season occurs between March and June. River discharge peaks in the latter months of the rainy season from September to November (Rivera-Monroy and Twilley, 1996).

The lagoon is almost completely bordered by extensive mangrove forests; whereas the river watersheds are located in agricultural and cattle feeding areas. In the lower catchments of the Palizada River, for instance, about 12,000 ha is used as rice fields and usage of pesticides (e.g. chlorpyrifos, malathion, parathion, carbofuran) is applied in the same fields (Rendón-Von Osten et al., 2006; von Osten et al., 2004). Dichlorodiphenyltrichloroethane (DDT) has been used in campaigns to control vectors of malaria: an endemic disease in this region (Benitez and Barcenas, 1996).

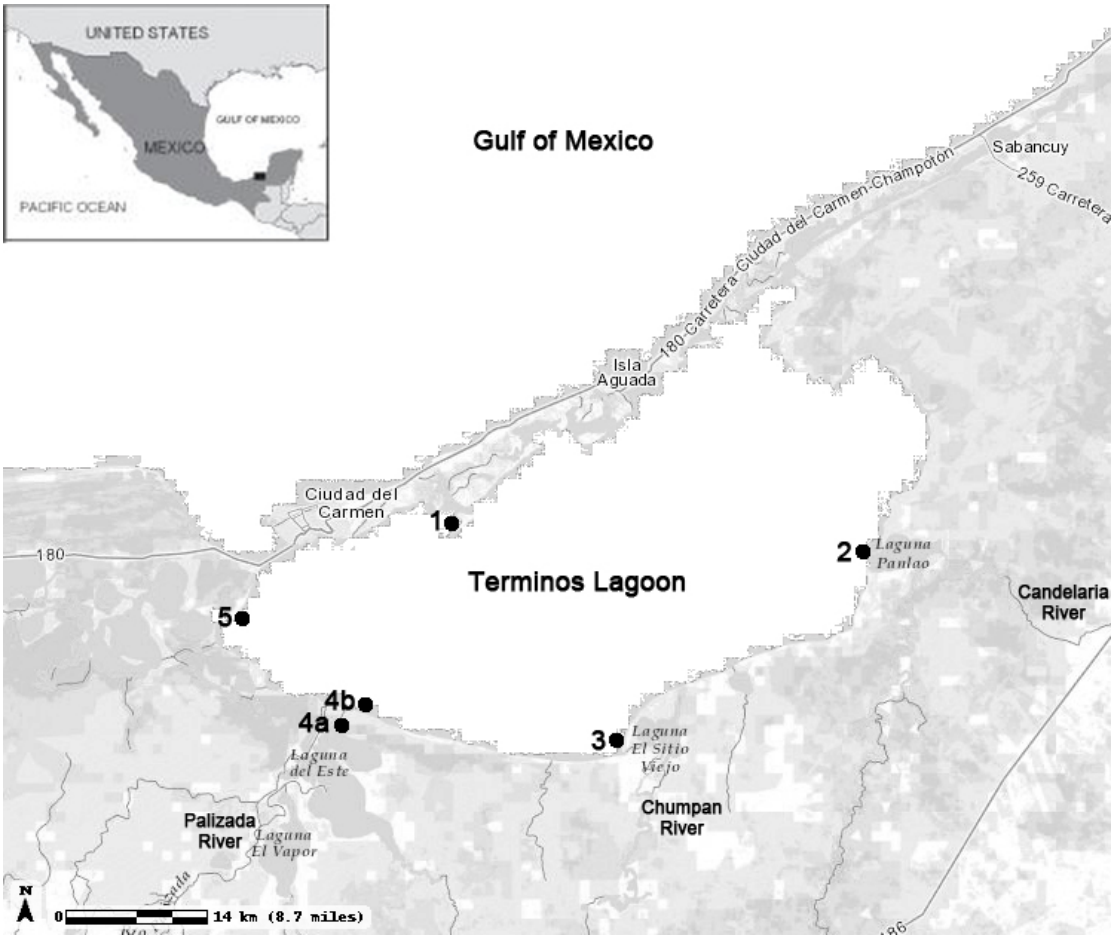


Figure 1. Map of Términos Lagoon and the transplantation sites. Biggest oyster (*Crassostrea virginica*) bank in the region located in Point 5: choosed site to collect the organisms for the experiment.

2.1.2 Champoton River

Champoton River is located in the southeastern Mexico and it is the main surface stream of the Yucatan Peninsula (**Fig. 2**). This coastal river has 48 km (length) and its outlet has a drainage basin surface area of 650 km^2. This river lacks tributaries to its flow, where the course can be divided into two portions: freshwater, salinity up to 1.2 practical salinity units (P.S.U.), and an estuary, salinity reaching 10–35 P.S.U. During the hurricane season the conditions may decrease salinity in the estuary (López-López et al., 2009).

The surrounding vegetation of the river consists of mangrove swamps in the lower reaches and medium to low perennial rain forest in the rest of the basin. The main anthropogenic activity in the basin is agriculture and, to a lesser extent, livestock raising (CONABIO, 1999). Champotón River exhibits several characteristics associated with ecosystems susceptible of conservation (López-López et al., 2009), however, it faces major challenges

from deforestation to non-point source pollution (Arriaga et al., 1998; Quetz et al., 2009; Rendón von Osten et al., 2008).

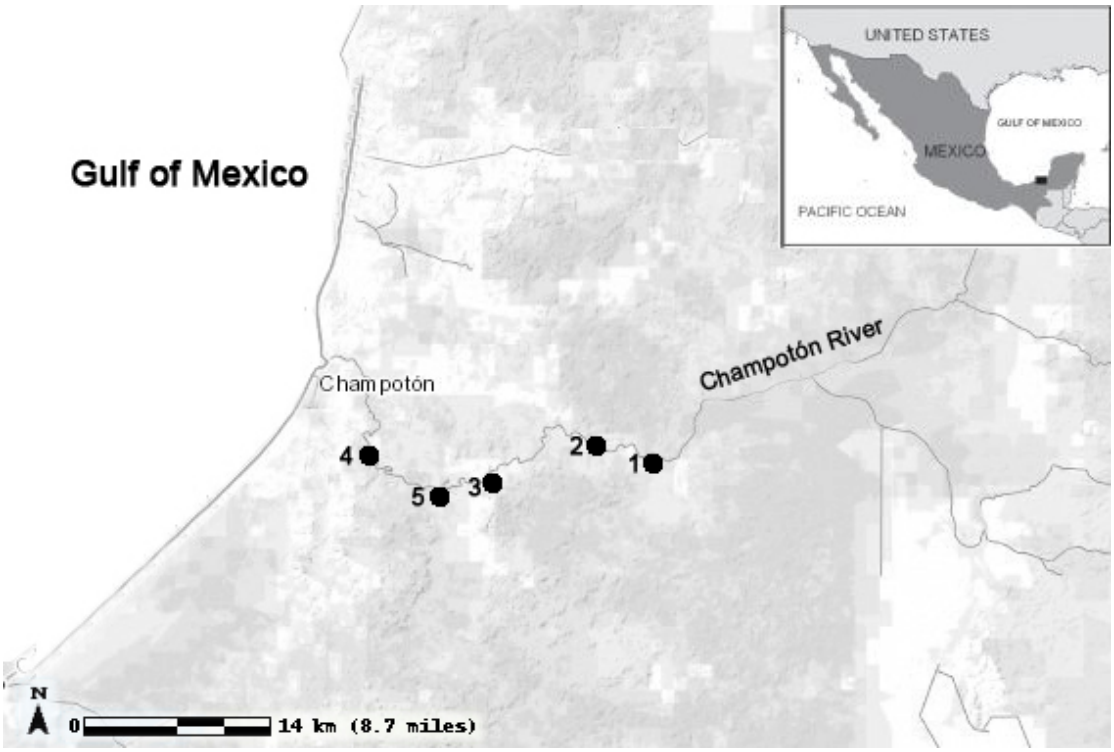


Figure 2. Map of Champoton River showing the subsequent transplantation sites. Clams (*Rangia cuneata*) were collected in Point 5.

2.2 Organism selection and transplantation

2.2.1 Términos Lagoon

The indigenous species *Crassostrea virginica* (n=1080) was collected at Point 5 in Términos Lagoon (November of 2011) and divided in 18 groups (n=60); each group was placed in a polypropylene (PP) mesh bag (80 mm). In each sampling point 3 bags to ensure viability along the experimental time. The criteria to choose points were their proximity to anthropogenic impact (Point 1) or different river inflow (Point 2, 3, 4a and 4b). In point 4 was occurring a dredge, so Point 4a is before the dredge and 4b after. Sampling periods should occur each 30 days, however, it varied according to the weather forecast, along a 4 months experiment. Each time ten organisms from one of the bags were collected per each sampling period for biomarker analysis. At some periods, we failed to retrieve some bags due to the weather or other external conditions (fisherman, currents, etc.).

2.2.2 Champoton River

The native species of Champoton River is the clam *Rangia cuneata*, where its only natural bank occurs at point 5. Two subsequent exposure periods of 3 months each took place: at October of 2011 and at January of 2012. Approximately 300 clams were sampled (point 5) and divided into groups (n=60); each group was placed in a polypropylene mesh bag (80 mm) and one or two bags were left at each point. In each sampling period 10 organisms were collected for biomarker analysis. At some periods, we failed to retrieve some bags due to the weather or other external conditions (fisherman, currents, etc.).

2.3 Environmental variables

In both study sites (*Terminos* and *Champoton*) physico-chemical parameters (temperature, salinity, pH, conductivity and dissolved oxygen) were measured at all periods and sampling points. Water was collected to quantify the chlorophyll-a and the nutrients (silicate, phosphate, ammonium and nitrate), in all sample points and all times. Chlorophyll-a and nutrients analysis were performed right after the sample field or within 48 hours. Samples for chlorophyll-a were filtered (Whatman GF/C glass filter, 47 mm), pigment extracted (90% acetone) and spectrophotometer analysis performed (Jeffrey and Humphrey, 1975). The analysis of nutrients (silicate, phosphate, ammonium and nitrate) was performed with the filtered samples and was determined by the methods described in UNEP/IOC/IAEA (1991). Data displayed in the supplementary material (**Tables 6, 7 and 8**).

2.4 Body Condition Index

Length, width and heights of the shells were recorded for each individual and used to calculate condition indices of the individual bivalve. The condition index was calculated according to the following equations:

Internal volume = 3/4 * length * width * height

Condition Index (CI) = Dry weight (g) / Internal Volume (cm^3)

The dry weight was obtained after the dissection of organisms; the whole soft tissue was dried at 45ºC for 48 hours and weighted.

2.5 Biomarker analysis

AChe, GST, CAT and SOD activities were determined in the digestive gland of the organisms. Tissue was homogenized in phosphate buffer (0.01 M, pH 7.4), centrifuged (20 min, 11500 rpm) (Howcroft et al., 2011), and then, the post-mitochondrial supernatant (PMS) used to determine the endpoints.

AChe was determined in the PMS, using 50 μL of sample and 250 μL of reaction buffer (30 mL K-Phosphate 0.1 M pH 7.2, 0.2 mL acetylcholine 0.075 M and 1 mL DTNB 10 mM). The activity was determined using an absorbance of 414 nm, following protocol described by Ellman, Courtney, Andres, & Featherstone (1961) and adapted to microplate by Guilhermino, Lopes, Carvalho, & Soares (1996). The absorbance was measured at 414 nm. Glutathione S-Transferase (GST) activity was measured at 340 nm, following the methodology of Habig & Jakoby (1981) and adapted to microplate by Frasco & Guilhermino (2002). GST was determined in 100 μL of PMS and based on the conjugation product of GSH and CDNB. CAT was determined by the method of Clairborne (1985), and its activity was evaluated by kinetic measurement following the decrease in absorbance at 240 nm due to H_2O_2 decomposition.

To measure the total Superoxide Dismutase (SOD) activity the method suggested by Suzuki (2000) was used. Absorbances were read in a microplate reader at 560 nm. The enzyme activity is expressed in units of SOD mg^{-1} protein. One unit of SOD is defined as the amount of enzyme required to inhibit 50% of the maximum reaction of $O_2^{\cdot -}$ with nitroblue tetrazolium (NBT). Protein concentration was determined according to the Bradford (1976) method, using bovine serum albumin as standard. Results are expressed in nmol min^{-1} mg $protein^{-1}$.

2.6 Statistical and data analysis

SPSS 21.0 software was used for all the statistical analysis. Data were tested for normality (Kolmogorov-Smirnov and Shapiro-Wilk tests) and

homogeneity (Levene's test), and did not pass the tests. Kruskal-Wallis test was performed and Mann-Whitney U post-hoc test was used to verify the differences between stations at the same period and between different periods per station. Pearson correlations were used to verify the influence of environmental factors on the physiology of bivalves and within physiological variables. Correlations were tested between physical-chemical parameters, nutrients and chlorophyll a vs. biomarkers responses and body condition index. A Principal Component Analysis (PCA) was also performed to discriminate sites. Mean values of biomarkers (AChe, GST, CAT, SOD) and CI were used for each station as variables in PCA analysis. To ensure equal treatment during Pearson correlations and PCA analysis all variables were standardized, the method chosen was the Z score, with a mean of zero and a standard deviation of one.

3. Results

3.1 Condition Index

3.1.1 Términos Lagoon

Condition index of *C. virginica* (**Fig. 3**) show significant increases over time in the Points 3, 4a, 4b and 5 against initial values (Kruskal Wallis: $p<0.01$), especially after 48 days of experiment. All stations differ amidst themselves at any sample period (Kruskal Wallis: $p<0.01$).

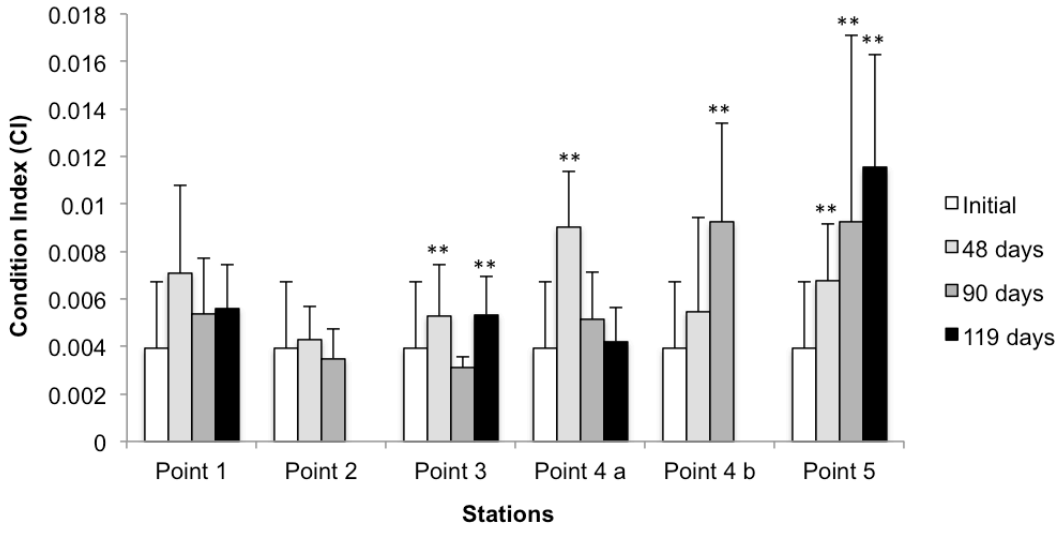


Figure 3. Condition index (mean ± standard deviation) of oysters *Crassostrea virginica* (n=10) in different stations of Términos Lagoon during 4 months. Statistical significance of data compared with the initial values (**< 0.01).

3.1.2 Champoton River

Condition Index of *R. cuneata* (**Fig. 4**) show slightly higher values in the dry season than rainy season. No significant differences ocurred between stations or time periods, in both seasons.

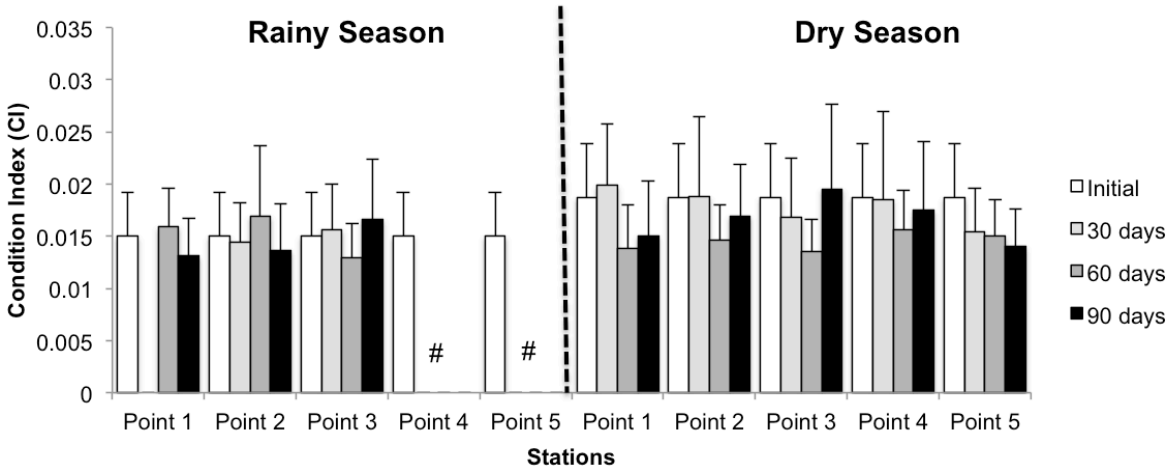


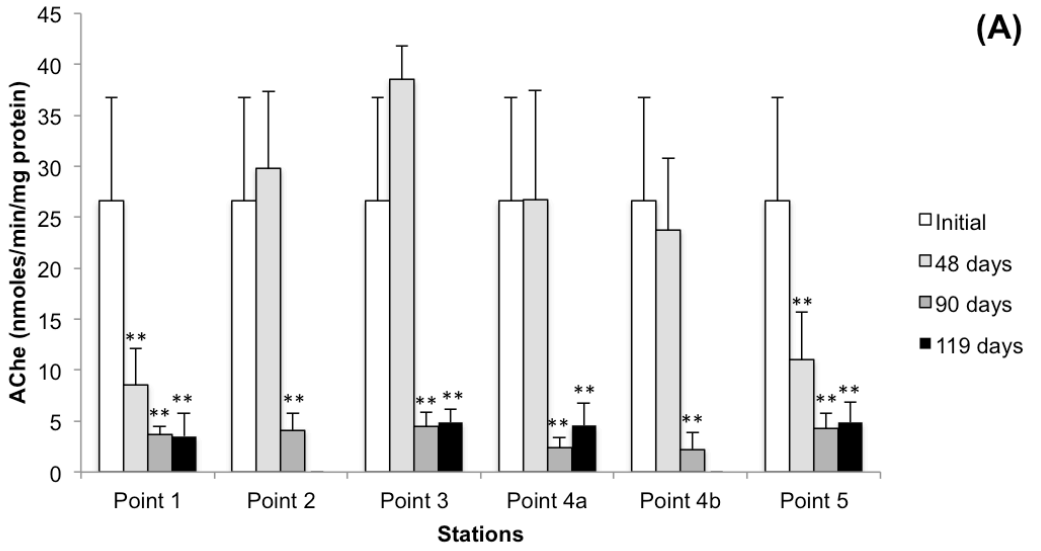
Figure 4. Condition index (mean ± standard deviation) of clams *Rangia cuneata* (n=10) in different stations of Champoton River during 2 seasonal experiments. # Means that the point was lost.

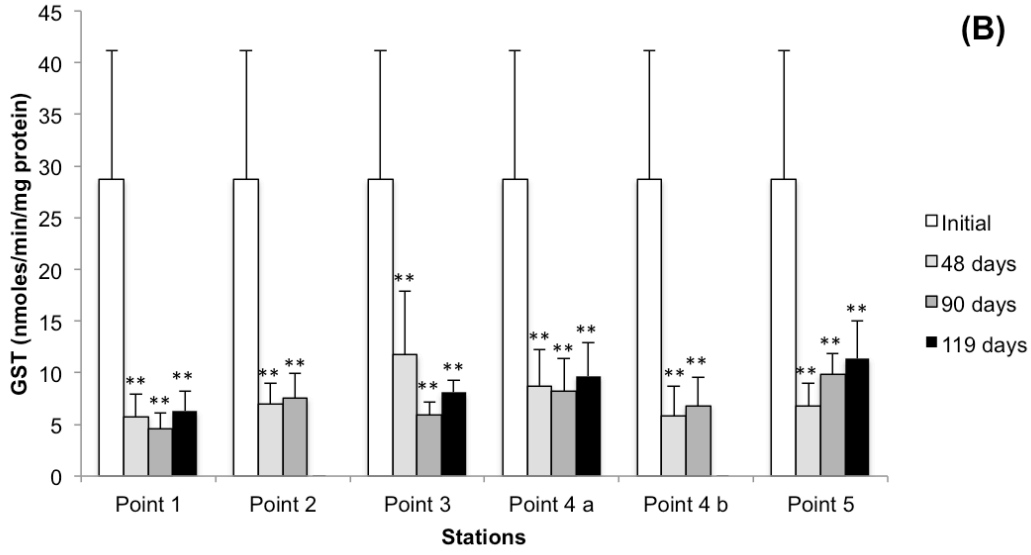
3.2 Biomarkers responses

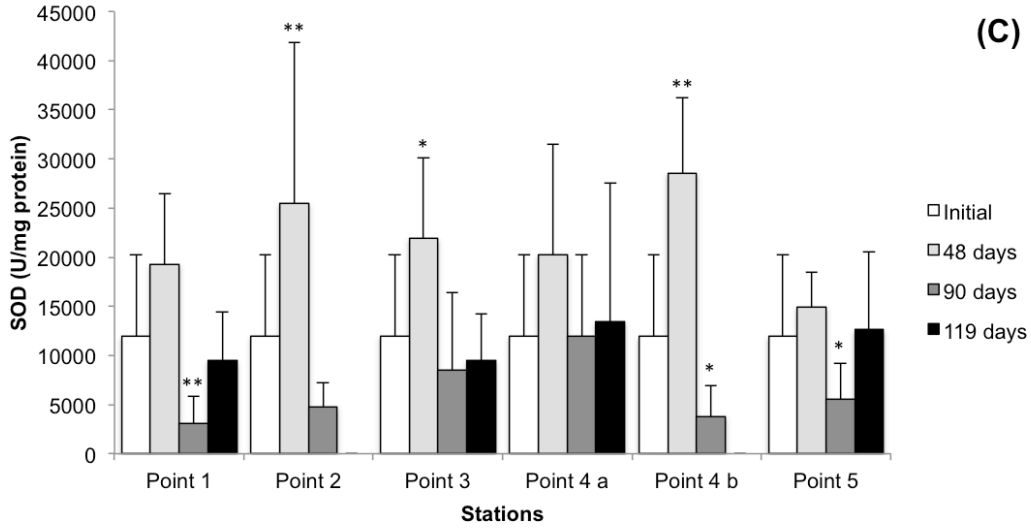
3.2.1 Términos Lagoon

Response of biomarkers (AChe, GST, SOD and CAT) shows a decrease in enzymatic activity over time (**Fig. 5**). Regarding neurotoxicity response, AChe (**Fig. 5A**) activity was highly inhibited of when compared with the initial values, mostly after 90 days of exposure (Kruskal Wallis $p<0.01$). At 48 and 90 days occurs differences among sites: all points differ among themselves, except Point 1 and 5 (Mann Whitney: $p<0.01$).

A clear inhibition of the GST activity (**Fig. 5B**) occurs against the initial values (Kruskal Wallis: $p<0.01$). Among points significantly differ at 90 and 119 days (Kruskal Wallis: $p=0.039$ and $p=0.007$), mostly between Point 1 and other points (Point 2, 3, 4a and 5).


(A)
AChe (nmoles/min/mg protein)
45
40
35
30
25
20
15
10
5
0
Point 1
Point 2
Point 3
Point 4a
Point 4b
Point 5
Stations
Initial
48 days
90 days
119 days


(B)
GST (nmoles/min/mg protein)
45
40
35
30
25
20
15
10
5
0
Point 1
Point 2
Point 3
Point 4 a
Point 4 b
Point 5
Stations
Initial
48 days
90 days
119 days


(C)
SOD (U/mg protein)
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Point 1
Point 2
Point 3
Point 4 a
Point 4 b
Point 5
Stations
Initial
48 days
90 days
119 days

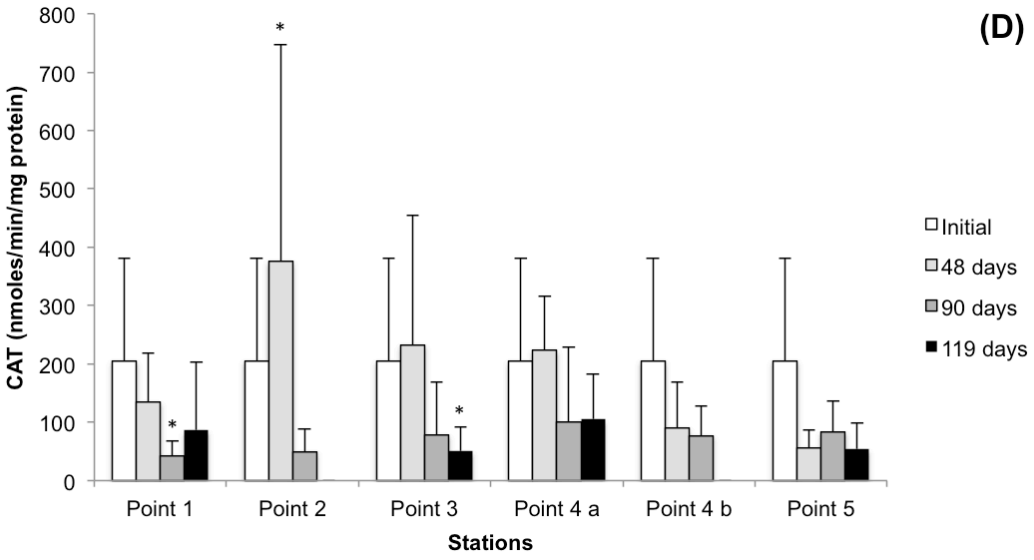


Figure 5. Biomarkers analyzed in digestive gland (mean ± standard deviation) of *Crassostrea virginica* (n=10) in different stations from Términos Lagoon: (A) Acetylcholinesterase (AChe), (B) Glutathione S-transferase (GST), (C) Superoxide dismutase (SOD) and (D) Catalase (CAT). Statistical significance compared with the initial values (* < 0.05 and ** < 0.01).

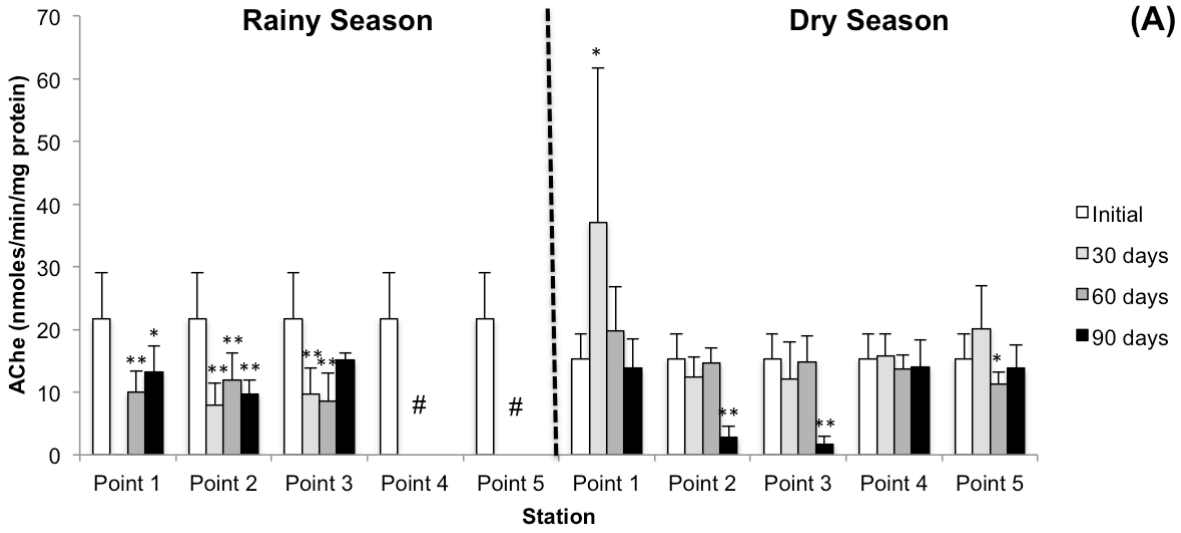
A small pattern was noticed in SOD activity (**Fig. 5C**): higher values after 48 days of experiment followed by an inhibition after 90 days (Kruskal Wallis $p<0.05$). The only contrast between stations happened after 90 days of transplant, where Point 4a differs from Points 1, 4b and 5 (Mann Whitney: $p=0.007$, $p=0.014$ and $p=0.047$, subsequently).

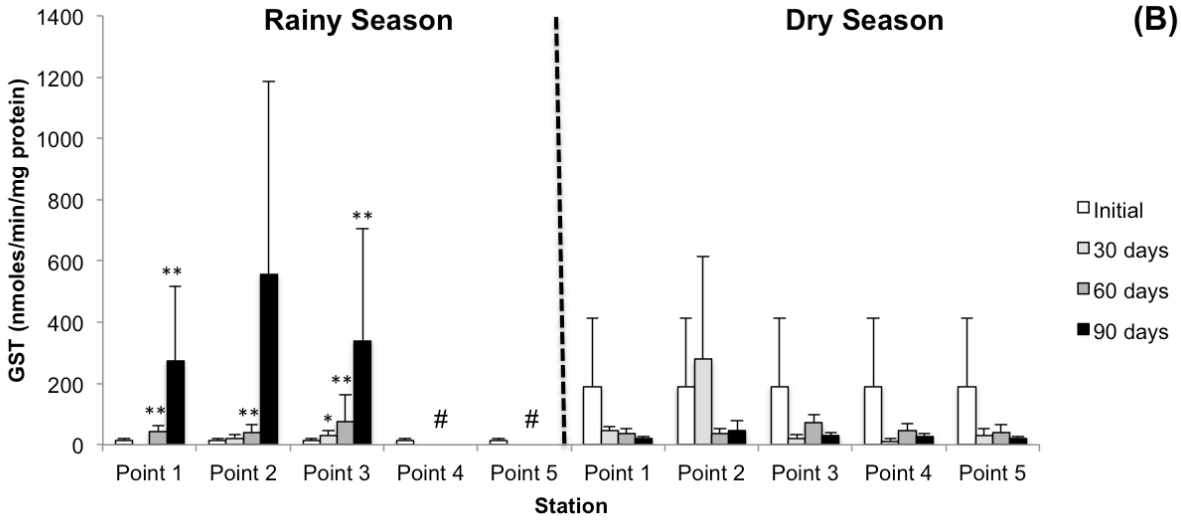
CAT activity (**Fig. 5D**) decayed over time against the initial values, except Point 2 at 48 days (Mann Whitney: $p=0.023$). Data is lacking a clear arrangement, whereas points at the distinct sampling periods, lack significant differences among each other.

3.2.2 Champoton River

The response of selected biomarkers (AChe, GST, SOD and CAT) shows different trends over time and season (**Fig. 6**). AChe activity (**Fig. 6A**) underwent an inhibition over time in all sites and both seasons; however, at rainy season this decline in the response is highly significant (Kruskal Wallis: $p<0.01$). Among points, differences are more pronounced in the dry season, differing amidst themselves. In the rainy season only Points 2 and 3 show differences after 90 days of transplant (Mann Whitney: $p=0.013$).

In GST (**Fig. 6B**), the rainy season clearly influences more the endpoint. At rainy season an exponential increase occurred over time with a peak after 90 days (Kruskal Wallis: $p<0.05$); stations lack differences among each other in any sample periods. In the dry season occurred an opposite trend: reduction of the GST activity and stations are distinct among each other in all sample periods (Kruskal Wallis: $p=0.002$, $p=0.025$, $p=0.023$ for 30, 60 and 90 days respectively).





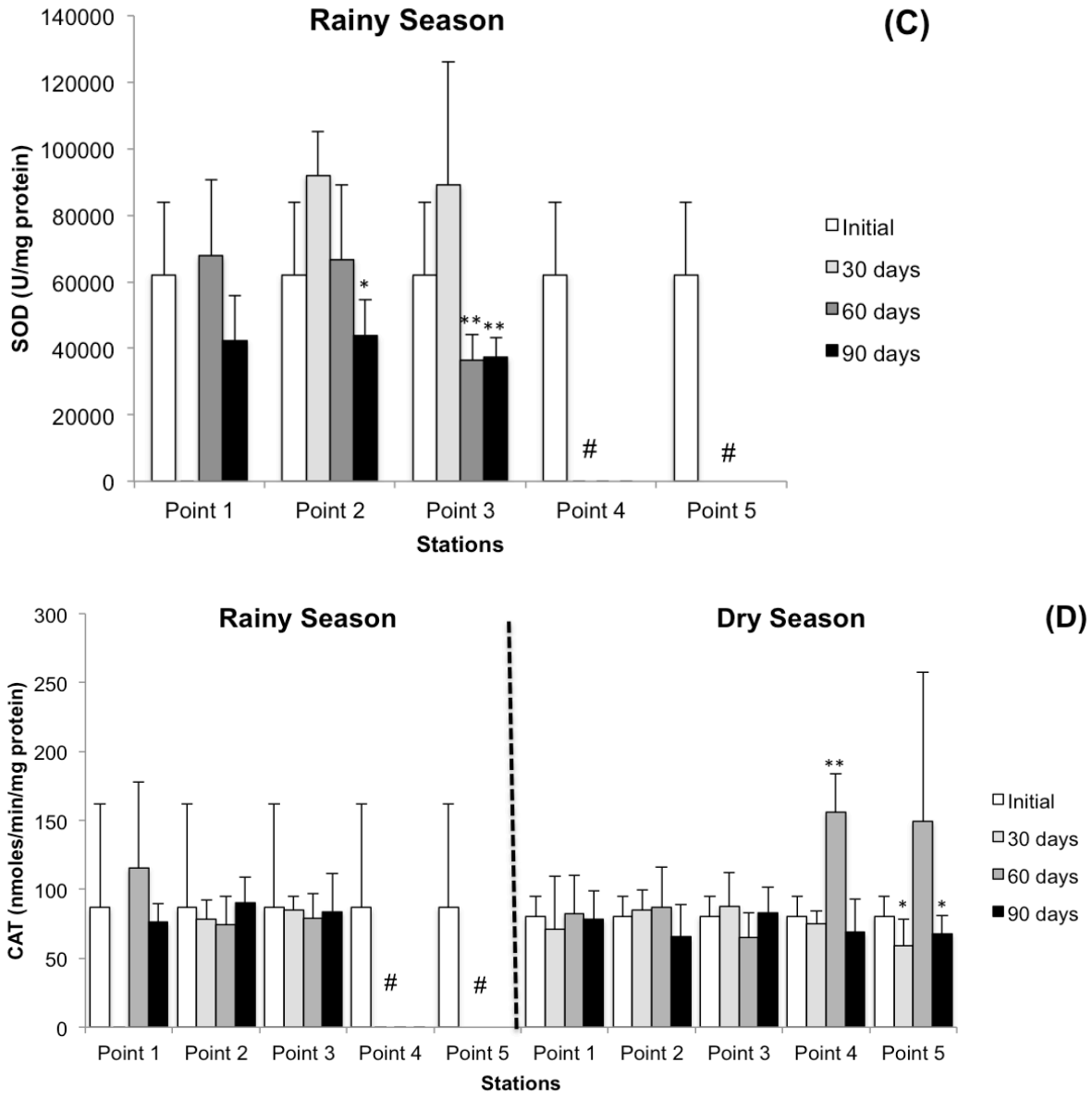


Figure 6. Biomarkers analyzed in digestive gland (mean ± standard deviation) of *Rangia cuneata* (n=10) in different stations from Champoton River: (A) Acetylcholinesterase (AChe), (B) Glutathione S-transferase (GST), (C) Superoxide dismutase (SOD) and (D) Catalase (CAT). Statistical significance of data compared with the initial values (* < 0.05 and ** < 0.01). # Means that the point was lost.

SOD was only detected at rainy season (**Fig. 6C**) with an inhibition trend over time, especially in Points 2 and 3 (Kruskal Wallis: $p<0.05$). Point 3 significantly differs from other sample points (Mann Whitney: $p=0.007$ and $p=0.002$, respectively). CAT activity (**Fig. 6D**) lacks significant trends and remained constant through both seasons, except for Points 4 and 5 in the dry season (Kruskal Wallis: $p<0.05$): inducted and an subsequently inhibited. Due

to this disparity, all other stations differ from Point 4 at this period (Mann Whitney: $p<0.01$).

3.3. Relationship between physiological responses and environmental variables

3.3.1 Términos Lagoon

Person correlation for the selected endpoints (biomarkers and CI) and physico-chemical parameters (**Table 1**) show an impact of abiotic factors in the response of organisms, especially in the neurotoxicity enzyme (AChe). AChe was greatly affected by temperature, conductivity, salinity, phosphate and silicate levels. Regarding oxidative stress enzymes (GST, CAT, SOD), temperature, salinity, levels of silicate and phosphate influenced the organism responses.

Table 1. Pearson correlation coefficient between biomarker responses in digestive gland of *Crassotrea virginica*, condition index (CI) and environmental variables in Términos Lagoon.

	AChe	GST	CAT	SOD	CI
Temperature	**-0.743****	**-0.663****	**-0.634****	-0.216	**-0.692***
Conductivity	**-0.460***	-0.374	-0.383	-0.332	-0.378
pH	0.005	-0.318	0.028	0.398	-0.258
Salinity	**-0.641****	-0.418	**-0.510***	**-0.485***	-0.396
DO (mg/L)	-0.366	-0.173	-0.308	-0.079	-0.141
Chlorophyll a	0.076	-0.234	0.007	0.390	-0.237
Silicate	**0.555****	**0.568****	**0.597****	0.214	**0.599****
Phosphate	**-0.681****	-0.407	**-0.530***	**-0.616****	-0.366
Nitrate	-0.338	-0.236	-0.346	-0.132	-0.258
Ammonium	0.230	**0.503***	0.032	-0.142	**0.518***

Data were pooled across sites. Values and asterisks in bold indicate significant relationships (*$p<0.05$, **$p<0.01$).

3.3.2 Champoton River

Pearson correlation (biomarkers response, condition index and physico-chemical parameters) shows an impact of abiotic factors in the response of selected endpoints, mainly in the rainy season (**Table 2**). SOD

was the most affected biomarker in the rainy season, followed by GST. In the dry season, the measured variables, lack effects on the endpoints response.

Table 2. Pearson correlation coefficient between biomarker responses in digestive gland of *Rangia cuneata*, condition index (CI) and environmental variables in Champoton River during rainy (R) and dry (D) seasons.

		AChe	GST	CAT	SOD	CI
Temperature	**R**	**0.793****	0.005	0.274	-0.462	-0.064
	D	0.098	-0.341	**0.662****	-	**-0.577****
Conductivity	**R**	-0.424	**0.600***	0.064	**-0.754****	-0.052
	D	-0.077	-0.150	0.080	-	-0.098
pH	**R**	-0.286	-0.328	0.240	**0.769****	0.046
	D	0.351	0.375	-0.035	-	0.144
Salinity	**R**	-0.231	0.534	0.055	**-0.818****	-0.182
	D	-0.084	-0.210	0.012	-	0.177
DO (mg/L)	**R**	**-0.756****	0.496	0.070	-0.395	-0.142
	D	0.126	-0.138	-0.025	-	-0.262
Chlorophyll a	**R**	-0.242	**0.715***	0.030	**-0.735***	-0.115
	D	-0.131	0.399	-0.273	-	**0.709****
Silicate	**R**	0.232	0.363	0.257	**-0.757***	-0.028
	D	0.087	-0.335	0.054	-	-0.244
Phosphate	**R**	0.267	**0.862****	-0.078	-0.528	0.274
	D	0.367	-0.192	-0.221	-	0.400
Nitrate	**R**	-0.232	0.516	-0.220	**-0.686***	0.010
	D	0.073	0.00	0.055	-	-0.140
Ammonium	**R**	0.848	-0.254	0.036	0.035	0.014
	D	**0.477***	-0.173	0.229	-	-0.359

Data were pooled across sites. Values and asterisks in bold indicate significant relationships (*p<0.05, **p<0.01).

3.4 Principal Component analysis

3.4.1 Términos Lagoon

The PCA (**Fig. 7 and 8**) indicate 2 principal components summarizing the set of variables (biomarkers and CI) and accounting for 92.51% of the total variance (**Table 3**). All endpoints influenced positively the first principal

component, accounted for 61.91% of the original variance. The second principal component explained 30.60% of the variance: negative values associated to GST and CI and positive values to CAT and SOD.

Table 3. PCA: Component loadings of the variables for the two principal components in Términos Lagoon.

Variables	*Component 1*	*Component 2*
Eigen values	*3.096*	*1.530*
% of variance	*61.91*	*30.60*
Ache	*0.924*	*-*
CAT	*0.853*	*0.334*
GST	*0.834*	*-0.534*
CI	*0.803*	*-0.570*
SOD	*0.418*	*0.853*

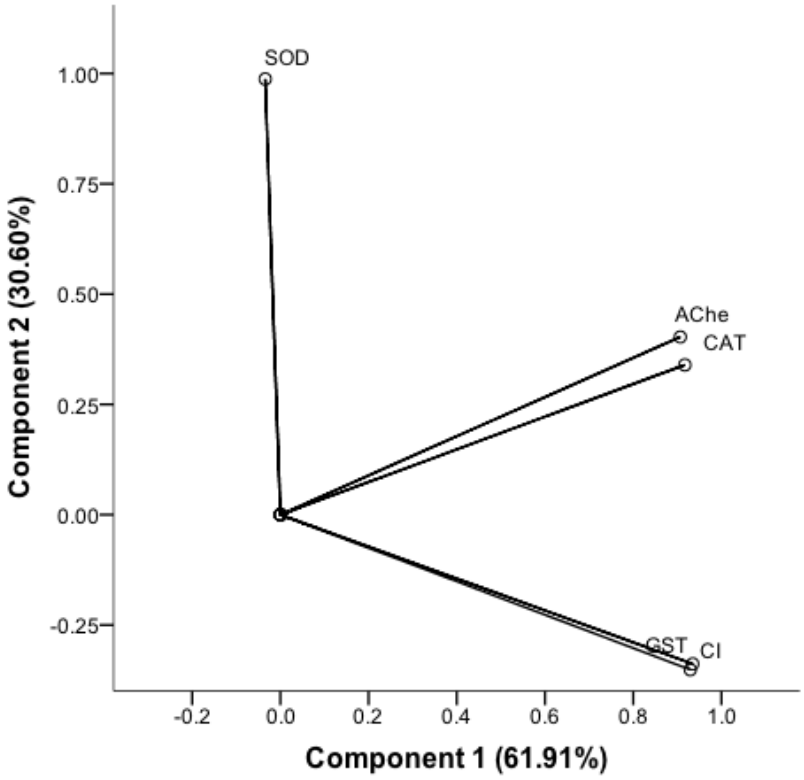


Figure 7. Plot of variable vectors for the two dominant components produced by biomarkers (AChe, GST, CAT, SOD) and condition index (CI) of Términos Lagoon.

Plot of scores for PC1 and PC2 for sample times and sites (**Fig. 8**) displayed a more time-dependent pattern, being more evident after 90 days of experiment. Although at 48 days of transplant data cluster, a site-dependency relation exists at this period– influenced by the GST and CI response (**Fig. 7**). The initial values stood apart from the other groups, being influenced mostly by SOD.

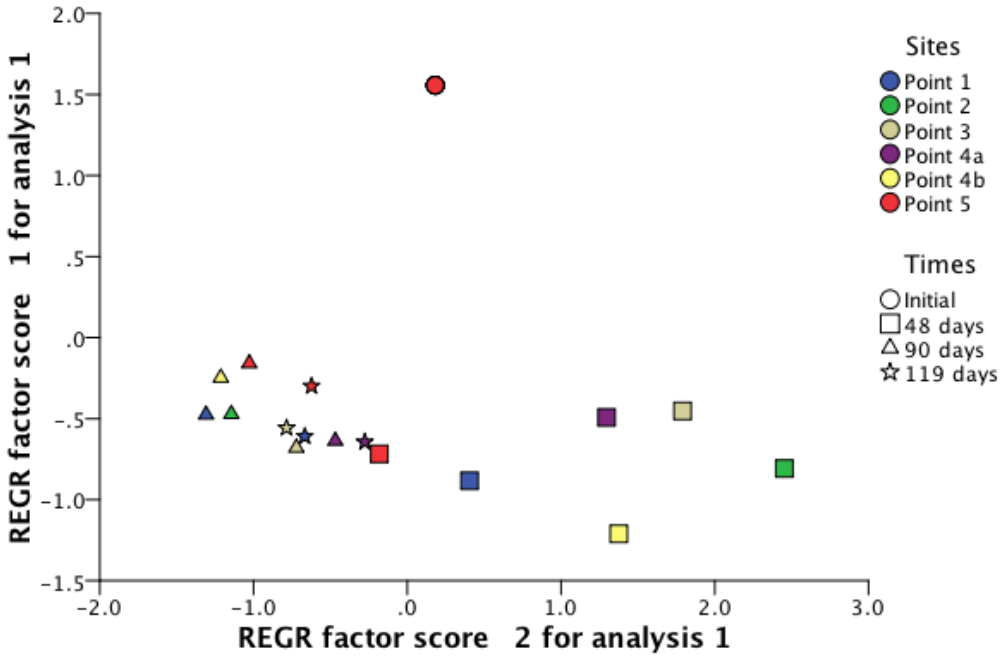


Figure 8. The distribution diagram of the different groups of sites during different experimental periods as a function of the two principal component axis. Principal component loading and total variance associated with each axis are provided in Table 3.

3.4.2 Champoton River

PCA results for the data from Champoton River at the rainy season (**Fig. 9 and 10**) suggest 3 principal components to explain the variables (biomarkers and CI), accounting for 79.72% of the total variance (**Table 4**). Principal Component 1 (PC1) undertaking 35.90% of original variance and undergoes through positive (SOD and CI) and negative (GST) influences. PC2 took account for 22.66% of the total variance and only positively influenced by GST, CAT and CI. PC3 explained 21.16% of variance: positive values linked to AChe and negative ones with SOD.

Table 4. PCA: Component loadings of the variables for the three principal components in Champoton River at rainy season.

Variables	*Component 1*	*Component 2*	*Component 3*
Eigen values	*1.795*	*1.133*	*1.058*
% of variance	*35.90*	*22.66*	*21.16*
SOD	*0.858*	*-*	*-0.354*
GST	*-0.786*	*0.480*	*-*
CAT	*-*	*0.692*	*-*
CI	*0.530*	*0.612*	*-*
AChe	*-*	*-*	*0.950*

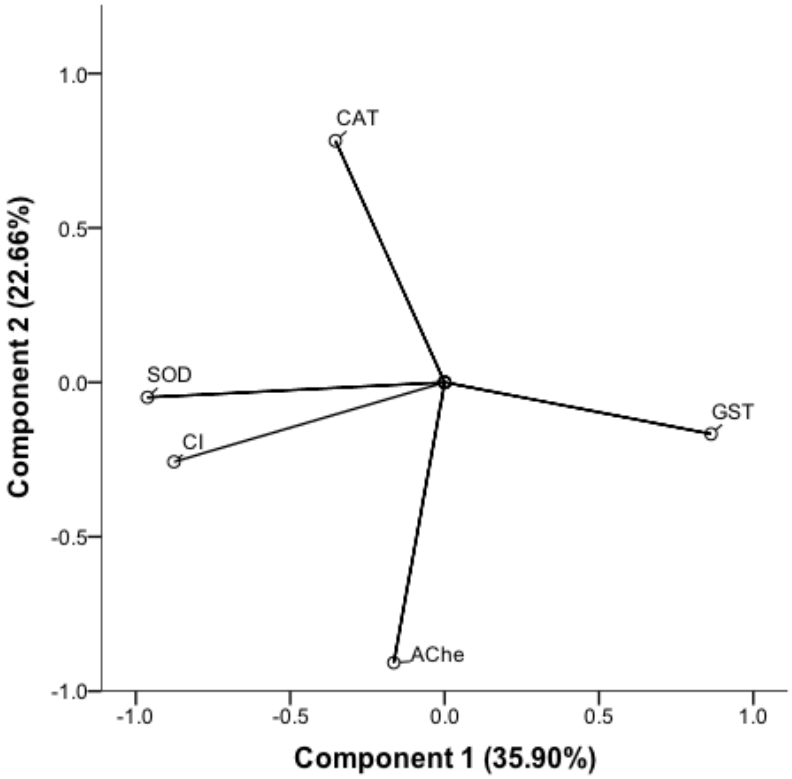


Figure 9. Plot of variable vectors for the two dominant components produced by biomarkers (AChe, GST, CAT, SOD) and condition index (CI) of Champoton River at rainy season.

The plot of scores of different sites for the two principal components (**Fig. 10**) lacks apparent trend. Each sample point responds differently over time. While at 30 days, points 2 and 3 grouped with initial values; after 60 and 90 days, a wide variety of responses occurred and each point is influenced by a different endpoint (**Fig. 9**).

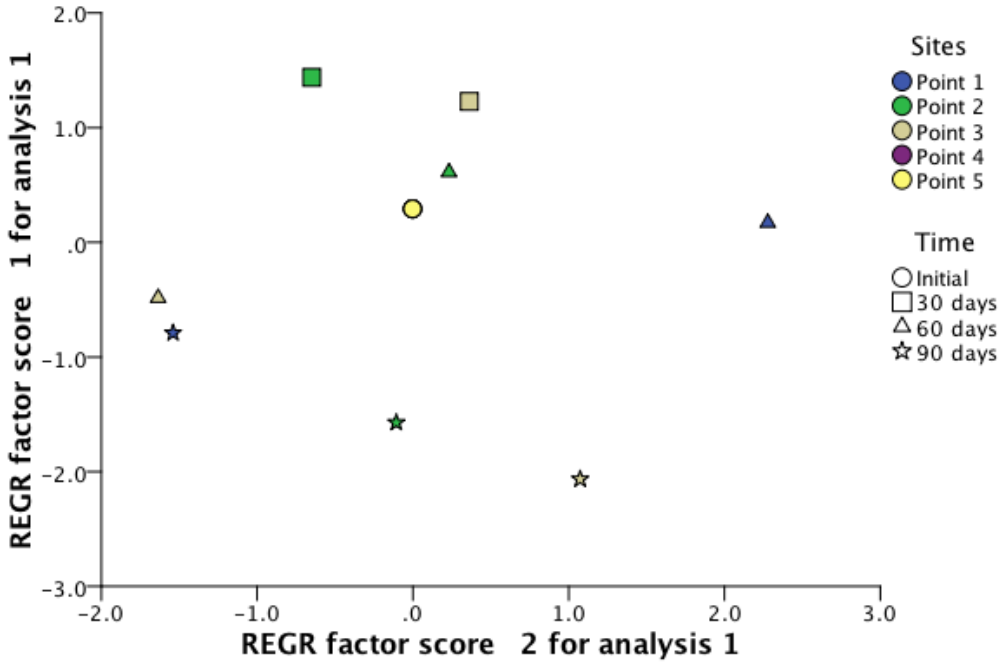


Figure 10. The distribution diagram of the different groups of sites during different experimental periods as a function of the two principal component axis in Champoton River at rainy season. Principal component loading and total variance associated with each axis are provided in Table 4.

The PCA for the data from Champoton River at the dry season (**Fig. 12**) resulted in two PCs that explained 77.17% of the total variance (**Table 5**). PC1 undertook 50.49% of the total variance and PC2 26.67%. Both PCs are influenced positively by AChe and GST; while CAT is negatively associated to PC1, CI is negatively related to PC2. This pattern is also revealed in the plot of variable vectors (**Fig. 11**).

Table 5. PCA: Component loadings of the variables for the two principal components in Champoton River at dry season.

Variables	*Component 1*	*Component 2*
Eigen values	*2.020*	*1.067*
% of variance	*50.50*	*26.67*
GST	*0.853*	*0.328*
CI	*0.745*	*-0.438*
Ache	*0.668*	*0.592*
CAT	*-0.540*	*0.646*

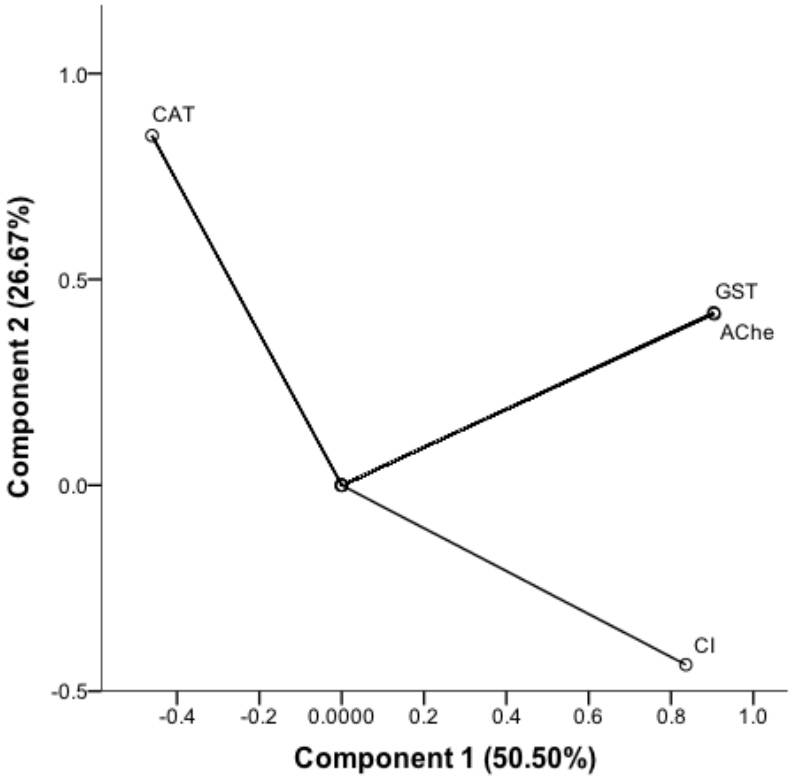


Figure 11. Plot of variable vectors for the two dominant components produced by biomarkers (AChe, GST, CAT) and condition index (CI) of Champoton River at dry season.

A plot of scores of different sites for the two PCs over transplantation periods (**Fig. 12**) showed a more time-dependent response of organisms in the dry season. At 30 and 90 days of transplant the overall response appears to be similar and influenced by CI, AChe and GST (**Fig. 11**). Although at 60 days this trend differs from 30 and 90 days, all points from this sample time

cluster together (except for Point 3). No major influences are linked to this trend shifting.

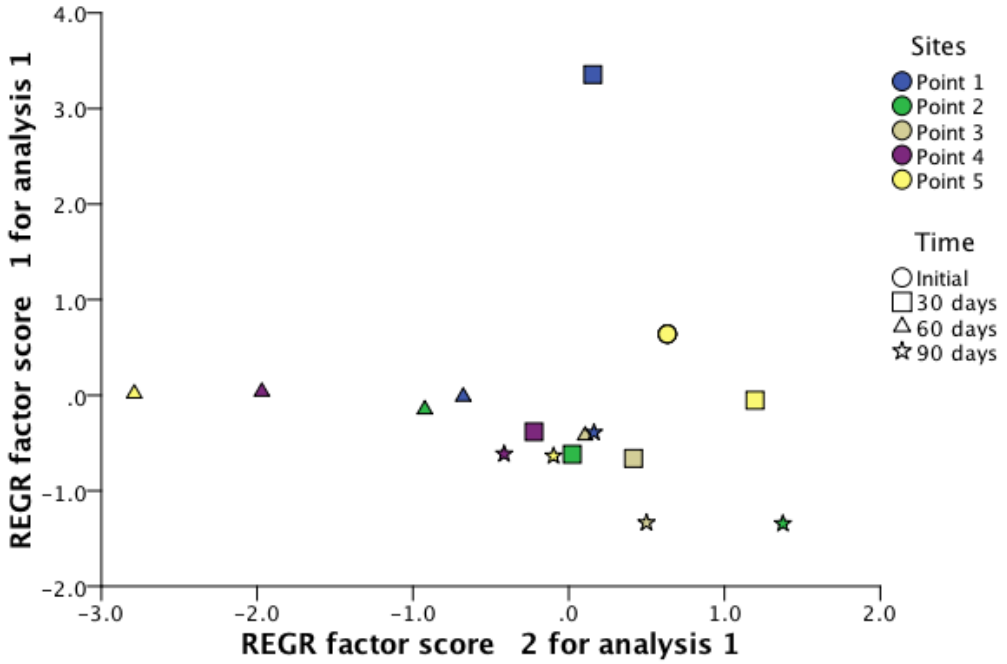


Figure 12. The distribution diagram of the different groups of sites during different experimental periods as a function of the two principal component axis in Champoton River at dry season.

4. Discussion

In this study, we aimed to compare the influence of different types of contamination (river inflow-related pesticides and anthropogenic impact-related hydrocarbons) in locations of known impact in the sublethal response of bivalves during a certain amount of time. We found a wide variety of responses in different compartments (Estuary and River). AChe was highly inhibited in Términos and the rainy season of Champoton; whereas, GST activity decayed in Términos and escalated in the dry season of Champoton. The oxidative stress response, represented by SOD and CAT, displayed different patterns in the distinct environments. Therefore, this result suggests an association between bivalves' response and the environmental contamination.

Comparing physiological responses of bivalves from different compartments (Estuary and River), apparently the overall response of

Términos and Champoton at dry season is more time-related than site specific. Both transplants overlap in time and were subject to similar weather impacts, explaining the match in their response.

4.1 Términos Lagoon

The inhibition of AChe in all stations was supported by several studies displaying the same trend (Campillo et al., 2013; Dellali et al., 2001; Escartín and Porte, 1997; Ochoa et al., 2013). These studies were performed in areas with intense agricultural activity and application of pesticides and biocides. Points 2, 3 and 4 (a,b) are closer to rivers, so they can be highly affected by pesticides and other types of agricultural waste. According to (Carvalho et al., 2009), pesticide residues traced in the biota were mainly chlorinated hydrocarbons and are primarily originate in the watershed of main rivers; thus probably our organisms were affected by pesticides resulting from applications in rice fields, being carried away by the river and finally, discharged into the lagoon.

Ache inhibition may also occur due to other contaminations from metals, domestic effluents, petroleum, PAHs, surfactants to other industrial pollutants (Bebianno et al., 2004; Choi et al., 2011; Payne et al., 1996). Point 1 and 5 were significantly different from other points, the higher proximity of the Ciudad del Carmen and anthropogenic pollution could explain this outcome. (Carvalho et al., 2009) postulated the origin of Polychlorinated Biphenyls (PCBs) and DDTs in oyster's tissues as most likely from the surrounded towns. Thus, our oyster and clams could be exposed to PCBs related to waste discharges from repair workshops and other industrial activities in the surrounded towns, and DDTs used in campaigns to control vectors of malaria (Benitez and Barcenas, 1996).

High water temperatures increase metabolic rates and consequently enhance the toxicity of anti-cholinesterase contaminants (Escartín and Porte, 1997; Moreira and Guilhermino, 2005; Ochoa et al., 2013). In our study, AChe values correlate with temperature, suggesting an effect of the abiotic factors (e.g. temperature, salinity) on the neuro-enzyme. Lehtonen et al. (2006): claimed that AChe inhibition implies general stress, being affected by changes in physico-chemical parameters

Concerning GST, the inhibition found in all periods lacks agreement with Van der Oost, Beyer, & Vermeulen (2003) theory: toxicity of many exogenous compounds can be modulated by induction of GSTs. Although some field studies with bivalves reported similar reduction in polluted areas (Bebianno et al., 2007; Cotou et al., 2013; Fernández et al., 2012; Regoli et al., 2004; Tsangaris et al., 2010); GST inhibition stands as a more unspecific response to the chemical challenge (Regoli et al., 2003), fitting in our case. Robillard, Beauchamp, & Laulier (2003) postulated a decay in GST activity in the presence of pesticides: another possible explanation for the high inhibition; whereas L. Vidal-Liñán, Bellas, Etxebarria, Nieto, & Beiras (2014) and Leticia Vidal-Liñán, Bellas, Campillo, & Beiras (2010) showed a lack of seasonal variability in GST: since in our outcomes temperature correlates with GST, it could affect the biomarker response. Sheehan & Power (1999) associated biomarker seasonal variation to the natural physiological cycle of bivalves; however, more studies relating biomarkers in tropical species and abiotic factors are required to develop a more elucidative theory.

Antioxidant enzymes, as superoxide dismutase (SOD) and catalase (CAT), regulate the content of ROS in aquatic organisms (Neumann et al., 2001). In our study, both enzymes follow the same pattern of inductions and inhibitions. Elevated levels of antioxidant enzymes imply a higher pollution degree (Cotou et al., 2013; Turja et al., 2013), explaining the Points 2, 3 and 4 situations. SOD catalyzes the dismutation of O_2^- to H_2O_2, being further degraded by CAT; thus, higher values of SOD indicate higher levels of H_2O_2, and supposedly, an increase in CAT activity, what could be the case in our study.

4.2 Champoton River

In Champoton River, season clearly affected the response of the organisms: especially in the rainy season when the effects in the endpoints were higher. AChe shows an inhibition at rainy season and in two points of the dry season. Several authors (Campillo et al., 2013; Kopecka et al., 2006; Ochoa et al., 2013; Tsangaris et al., 2010) associated the decline in the enzymatic activity to the presence of contaminants in field transplants. As postulated in the previous section, this trend could be associated with the

presence of pesticides or urban runoff; since Rendón von Osten, González, Memije, & Quetz (2008) and Quetz, Memije, Benítez, & Rendón von Osten (2009) found several PAHs and pesticides in the Champoton River and its sediments. Furthermore, (Rendón von Osten et al., 2008) also found seasonal variations for PCBs and hexachlorocyclohexanes, reaching their highest values during rainy season – matching our results for AChe.

Rainy season was more affected by abiotic factors, thus affecting the organism response. Dellali, Gnassia Barelli, Romeo, & Aissa (2001) studied the influence of seasonal differences in the Ache activity in *Mytilus galloprovincialis*, and associated it with changes in water temperature; this outcome supports our results, since Ache correlates with temperature in the rainy season.

GST activity exponentially and highly inducted over the rainy season, lacking trends or remarkable results in the dry season; Trujillo-Jiménez, Sedeño-Díaz, Camargo, & López-López (2011) supports this pattern with the fish *Astyanax aeneus* exhibiting similar one. GSTs can be induced by diverse contaminants, namely PAHs, PCBs, furans, phenobarbital compounds and others (Cunha et al., 2005; Hartl et al., 2007; Van der Oost et al., 2003). According to some studies (López-Hernández et al., 2007; Mestre R., 1997; Sedeño-Díaz and López-López, 2007; Trujillo-Jiménez et al., 2011) the water quality in rivers of México improves over the rainy season, however our levels of GST do not suggest it.

The antioxidant enzyme CAT displayed a lack of response in general, except for Points 4 and 5 at dry season. Point 4 is close to a village, whereas Point 5 used as balneary place; both places might be affected by a point source of anthropogenic pollution, thus should be frequently monitored. CAT pollution-related responses differ – increasing, decreasing or unchanging – in bivalves exposed to contaminants in the field or laboratory conditions (Cossu et al., 1997; Cotou et al., 2013; Livingstone, 2001; Regoli et al., 2004).

Contrary to CAT, SOD was inhibited in Points 2 and 3: also observed by Downs, Shigenaka, Fauth, Robinson, & Huang (2002) and Falfushynska, Gnatyshyna, & Stoliar (2013) and associated with polluted sites, these points are close to fish aquacultures what could bring several xenobiotics to the water. Inhibition of antioxidant enzymes is related to a deficiency of the

system (Cossu et al., 1997), a probable hypothesis for our results. According to (Trujillo-Jiménez et al., 2011), the major sources of pollution in this river influencing our overall results are: non-point sources from agriculture, livestock-related chemical residues and input of organic matter from small human settlements near the river. Additionally, high episodic loadings of contaminants have been detected in aquatic ecosystems following flooding events in the rainy season in the Champoton area (Adams et al., 2003).

4.3 General Comparison

High general difference between environments and seasons is observed in the same compartment - River. Terminos and Champoton River behaved similarly at dry season. However, SOD lacked analyzes for the river samples at the dry season, which might influence the overall response; so comparisons should be careful and not considered definitive. Discrimination between estuary and river revealed to be challenging, but we can establish link based on the similarities of bivalves' physiology. Higher endpoints batteries should be tested and transplants should be carried out in parallel times, nonetheless *in situ* experiments are problematic to maintain and should be constantly monitored - raising logistics difficulties. To overcome these limitations in tropical ecosystems similar studies should be performed, leading to a deeper knowledge of the physiological behavior of bivalves in these regions.

5. Conclusion

The responses of the organisms from Términos Lagoon and Champoton River strongly differ when comparing the whole experiments. The Principal Component Analysis from Términos and Champoton in the dry season show some similarities in the organism responses: initial values differ from other points and sample periods, but points and sample periods resemble more after the second sample period (48 days for Términos and 30 days for Champoton). These similarities are possibly related to a closer timeline to the experiments, explaining the seasonal disparities in Champoton and Términos experiments. These patterns have some exceptions potentially linked to seasonal variations or physiological responses of the organisms. In

both scenarios occurred, stress responses; however, this sort of experiment with bivalves was realized for the first time in both places. Therefore, we experienced difficulties to establish a decisive response and a conclusive link to xenobiotics. Further studies should take place with a higher battery of endpoints to better understand the physiology of organism in these places, which could elucidate the relationship between local pollution and enzymatic response.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

Authors acknowledge Fundação para Ciência e Tecnologia (FCT - Portugal) by the financial support through the grant SFRH/BPD/62561/2009 (Jessica C.L. Ladewig), Departament of Biology of Aveiro University and EPOMEX Center (Centro de Ecologia, Pesquerías y Oceanografía del Golfo de México).

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SUPPLEMENTARY MATERIAL

Table 6. Environmental parameters measured monthly from Point 1 to 5 in Términos Lagoon between November 2011 and March 2012. T-temperature, C – conductivity, S - salinity, DO – dissolved oxygen, Chl-a – chlorophyll-a, Sil – silicate, Phos – phosphate, Nitr – nitrate, Amm - ammonium.

Points	Month	T (ºC)	C (µS/cm)	pH	S	DO (mg/L)	Chl-a (mg/cm^3)	Sil (µmol/L)	Phos (µmol/L)	Nitr (µmol/L)	Amm (µmol/L)
1	Nov 11	24	42000	7.87	10	3.38	4.018	5356	50.98	856	234.9
	Jan 12	25.1	50400	8.79	20.47	3.91	20.16	1837	50.51	32377	123.5
	Feb 12	26.3	58700	8.51	24.28	4.22	18.66	1779	508.1	25579	176.4
	Mar 12	28.5	62100	8.15	24.2	4.1	16.81	1014	602.5	2971	177.8
2	Nov 11	23.5	13440	8.23	5.94	6.53	7.56	6873	47.29	1057	242.5
	Jan 12	24.9	20410	8.75	8.1	7.63	8.51	6570	42.36	11531	155.4
	Feb 12	26.2	36300	8.32	25	6.87	13.6	1452	526	6848	233
	Mar 12	-	-	-	-	-	-	-	-	-	-
3	Nov 11	23.1	14470	8.16	6.43	7.01	14.79	5172	49.14	704.9	313.8
	Jan 12	25.3	34500	8.1	0.97	0.15	20.98	2706	46.16	1208	138.2
	Feb 12	26.4	43800	8.31	17.8	7.34	23.64	2236	956.8	37160	210.6
	Mar 12	30.1	52200	8.17	20.08	11.23	6.41	1162	485.5	31118	166.3
4a	Nov 11	23.1	3120	8.36	1.35	6.43	10.02	6356	52.21	1611	304.7
	Jan 12	25.2	600	10.05	0.24	7.46	22.01	4944	46.16	3877	192.2
	Feb 12	27.5	6870	9.15	2.42	8.76	18.54	4799	585.5	10775	163.5
	Mar 12	31	18650	8.82	7.09	7.44	6.61	3747	481.3	5186	197.6
4b	Nov 11	22.5	716	9.15	0.39	6.11	8.35	6291	51.59	3877	152.3
	Jan 12	25.8	629	9.84	0.25	8.77	24.54	4979	47.79	12689	222.2
	Feb 12	27.3	2770	9.28	2.67	7.74	18.06	7902	1881	20796	186.7
	Mar 12	-	-	-	-	-	-	-	-	-	-
5	Nov 11	23	5410	8.23	2.64	7.09	9.58	6806	54.66	2266	256.8
	Jan 12	25.6	9640	8.21	3.28	9.28	29	3981	45.6	9768	300
	Feb 12	27.9	43700	8.39	17.81	8.09	16.52	1995	1704	7855	190.1
	Mar 12	30.3	43500	8.61	16.36	8.18	3.35	1940	623.5	7150	162

Table 7. Environmental parameters measured monthly from Point 1 to 5 in Champoton River between September 2011 and January 2012 (Rainy Season). T- temperature, C – conductivity, S - salinity, DO – dissolved oxygen, Chl-a – chlorophyll-a, Sil – silicate, Phos – phosphate, Nitr – nitrate, Amm - ammonium.

Points	Month	T (ºC)	C (μS/cm)	pH	S	DO (mg/L)	Chl-a (mg/cm^3)	Sil (μmol/L)	Phos (μmol/L)	Nitr (μmol/L)	Amm (μmol/L)
1	Sep 11	28.1	899	8.55	0.23	5.36	-	-	-	-	-
	Oct 11	25	544	9.2	0.18	5.77	3.60	6703	44.90	196.37	ND
	Dec 11	27.4	2240	8.75	0.77	6.91	10.1	6865	47.16	1546	ND
	Jan 12	27.6	2250	8.1	0.9	6.95	14.11	6865	316.3	1792	100.4
2	Sep 11	28.2	897	8.43	0.38	5.34	-	-	-	-	-
	Oct 11	25	557	9.36	0.17	5.76	3.47	6715	43.57	411.93	ND
	Dec 11	26.8	2250	8.05	0.7	6.64	8.99	6856	50.04	4824	27.7
	Jan 12	27.8	2230	8.32	0.79	6.87	12.3	6856	296.2	4553	76.05
3	Sep 11	28.1	1082	8.13	0.56	4.95	-	-	-	-	-
	Oct 11	25.8	780	8.87	0.28	5.5	5.50	6751	44.23	709.97	ND
	Dec 11	26.7	2530	8.2	0.96	6.57	10.58	6836	51.74	5141	27.7
	Jan 12	27.1	2530	8.2	0.89	6.3	14.7	6836	1303	4491	82.48
4	Sep 11	28.7	1280	8.28	0.68	4.53	-	-	-	-	-
	Oct 11	25.3	1093	8.52	0.41	5.06	5.2	6758	42.91	790.5	ND
	Dec 11	-	-	-	-	-	-	-	-	-	-
	Jan 12	26.3	3090	8.18	1.09	6.25	15.6	-	2130	4584	112.4
5	Sep 11	28.5	1086	8.63	0.54	4.77	0.86	6817	47.53	191.34	13365
	Oct 11	25.4	818	8.74	0.3	5.52	3.53	6750	45.55	845.92	ND
	Dec 11	26.4	2560	8.26	0.86	6.81	7.83	6853	52.33	5060	69.24
	Jan 12	27.4	2530	8.6	0.84	5.96	11.64	6853	2067	4645	82.28

ND – Not Detectable.

Table 8. Environmental parameters measured monthly from Point 1 to 5 in Champoton River between September 2011 and January 2012 (Dry Season). T- temperature, C – conductivity, S - salinity, DO – dissolved oxygen, Chl-a – chlorophyll-a, Sil – silicate, Phos – phosphate, Nitr – nitrate, Amm - ammonium.

Points	Month	T (ºC)	C (µS/cm)	pH	S	DO (mg/L)	Chl-a (mg/cm^3)	Sil (µmol/L)	Phos (µmol/L)	Nitr (µmol/L)	Amm (µmol/L)
1	Jan 12	27.6	2250	8.1	0.9	6.95	14.1	6865	316.3	1792	100.4
	Feb 12	27.7	2190	8.24	0.71	6.39	7.61	7672	2059	4932	204.6
	Mar 12	28.2	2162	8.39	0.72	6.29	3.54	7614	745.9	4332	162.1
	Apr 12	26.9	2149	8.48	0.81	6.31	3.22	7633	675	278	100
2	Jan 12	27.8	2230	8.32	0.79	6.87	12.3	6856	296	4553	76
	Feb 12	27.6	2210	8.2	0.76	6.35	13.4	7633	2311	4639	146
	Mar 12	27.9	2210	8.24	0.73	6.19	3.30	7671	581.5	4832	183.6
	Apr 12	26.8	2179	7.97	0.79	6.21	15.1	6788	591	4332	76.05
3	Jan 12	27.1	2530	8.2	0.89	6.3	14.7	6836	1303	4491	82.48
	Feb 12	28	2490	8.17	0.82	6.27	12.1	7422	2109	4284	190.1
	Mar 12	28.5	2490	8.1	0.8	5.95	1.96	7461	611	4886	189
	Apr 12	27.1	2500	7.87	0.79	6.22	7.74	7000	579	4832	82.48
4	Jan 12	26.3	3090	8.18	1.09	6.25	15.6	-	2130	4584	112.4
	Feb 12	28.2	2520	8.22	0.83	6.25	10.8	7441	2170	3990	183.1
	Mar 12	28.9	3000	8.12	0.98	6.85	4.83	7672	588	4487	155
	Apr 12	28	3470	7.92	1.14	6.08	8.72	6788	554	4886	112.4
5	Jan 12	27.4	2530	8.6	0.84	5.96	11.64	6853	2067.5	4645	82.28
	Feb 12	28.2	3130	8.18	1.06	6.27	9.35	7671	2072	4190	157.4
	Mar 12	30	2520	8.11	0.81	6.71	4.27	7038	586	4459	172.64
	Apr 12	27.7	2540	8	0.8	6.03	4.26	7038	609	4486	82.28

CHAPTER

3

BIOCHEMICAL RESPONSE OF A NON-INDIGENOUS CLAM (*CORBICULA FLUMINEA*) AND MUSSEL (*MYTILUS GALLOPROVINCIALIS*) TO A TRANSPLANT EXPERIMENT IN DIFFERENT SCENARIOS IN PORTUGAL

Biochemical responses of a non-indigenous clam (Corbicula fluminea) and mussels (Mytilus galloprovincialis) to a transplant experiment in different scenarios in Portugal.

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This chapter was submitted as an original article to:

Ecotoxicology

ABSTRACT

Transplants and experiments in situ are nowadays used to linked effects observed in laboratory assays to the reality of the environment and aquatic ecosystems. A battery of biomarkers was chose to assess the sublethal response of mussels (*Mytilus galloprovincialis)* and clams (*Corbicula fluminea*) at a certain amount of time (3 months of experiment) To the mussels it was chosen five points in the Aveiro Estuary, and for clams the Minho River was selected due to the high abundance of this non-indigenous species. The endpoints preferred were biomarkers of neurotoxicity (Ache, Bche, PrChe), oxidative stress (GST, CAT, GR and LPO) and Condition Index (CI). The endpoints for Aveiro Estuary demonstrate that Ache, GST, CAT and GR have very similar patterns and are related in the statistical analysis. The same did not occur in the experiment in the river, however Ache, GST and CAT have closer responses and could be related in some way in the statiscs. The Principal Component Analysis demonstrate for both experiments that different groups were formed, especially after 90 days of transplant, this could be an adaptation to the new location, detoxification or seasonal variability affecting the organisms responses. To better understand this selected mechanisms an experiment with a higher amount of time should be performed to see the fluctuations due to the weather and separate this to the effects of contaminants. Only in this way a link could be make through all the data sampled in the bioassays.

Keywords: *Mytilus galloprovincilais*, *Corbicula fluminea*, transplantation, biomarkers, Aveiro Estuary, Minho River.

1. Introduction

Over the last decades, biomarkers at suborganismal levels of organization (biochemical, physiological, and histological) have been considered to be a viable measurement of responses to stressors (Huggett et al., 1993). Many researchers have been using physiological parameters (i.e. biomarkers, scope for growth, etc.), in which those based on responses at molecular and cellular levels represent the earliest signals of environmental disturbance and are commonly used for monitoring nowadays.

Under field conditions, organisms are exposed to a multiplicity of chemical and physical stressors, against a background of naturally occurring seasonal fluctuations that, in and of themselves, are potentially stressful to the organisms. Biomarkers have the potential to act as integrative measures at the suborganismal level to indicate adverse conditions, whether natural or not, preceding population-level effects (Mayer et al., 2002).

The influence of some environmental factors have been studied in an attempt of elucidate the behavior of bivalves in alter many characteristics (metabolic rate, biomass, etc.) and in concentrate pollutants in their bodies. These factors include temperature, salinity, season and organic matter (Hagger et al., 2010; Vercauteren and Blust, 1996), body size (Mubiana et al., 2006; Sokolowski et al., 2004; Wang and Fisher, 1997; Zhong et al., 2013), sex and reproductive status (Liu et al., 2014; Richir and Gobert, 2014; Widdows and Donkin, 1991), tidal height (Lobel and Wright, 1982) and physiological condition (Marsden et al., 2014; Nilin et al., 2012; Widdows and Donkin, 1991). However, effects of these factors in quantitative terms still remain unclear with different results reported depending on the study, element, location or season (Rainbow, 2002).

In this study we aimed to compare the influence of different types of contamination (rivers inflow-related pesticides and- anthropogenic impact-related hydrocarbons) in locations of known impact in the sublethal response of bivalves during 4 months. We also aimed to assess if the response is similar in different environments: estuary (Aveiro Estuary) and river (Minho River). We hoped to improve the knowledge of *in situ* experiments in temperate environments and the physiological responses of bivalves to

environmental contamination in this region.

2.Materials and Methods

2.1 Study sites

2.1.1 Aveiro Estuary

The Estuary of Aveiro (**Fig. 1**) was chosen to realize the transplant with mussels (*Mytilus galloprovincialis*). The area of the Aveiro bay is around 1.7 km^2 and receives a freshwater input from Antuã River (15 m^3 s^{-1}). The water height in the bay varies from 1 to 4 m with the tide. During spring tides, approximately 75% of the water bay is renewed, implying the export of anthropogenic material discharged to the bay (Hall et al., 1987) and of contaminated sediments re-suspended by the tidal currents (Pereira et al., 1998).

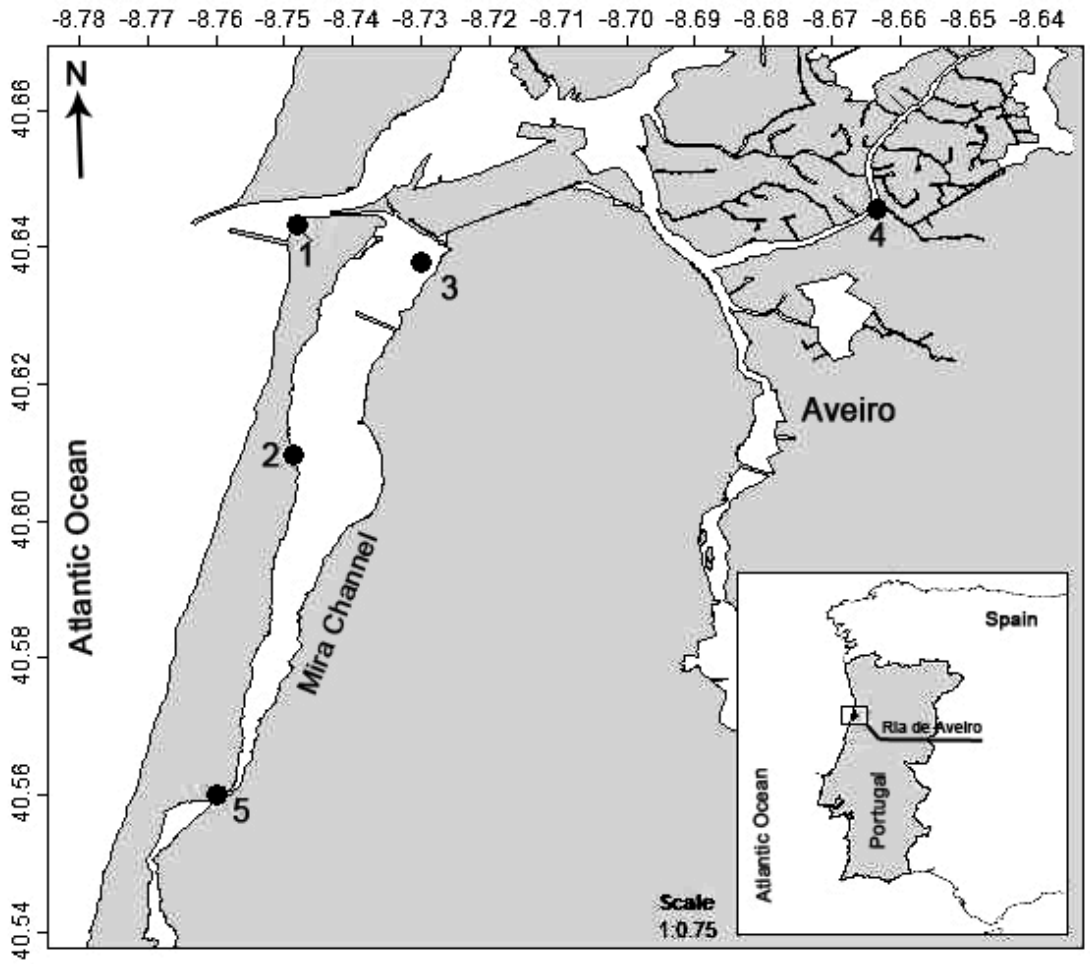


Figure 1. Map of Aveiro Estuary and the transplantation sites. Point 1 has the biggest mussel (*Mytilus galloprovincialis*) abundance in the region and it was the site choosed to collect the organisms for the experiment.

2.1.2 Minho River

The freshwater ecosystem chosen to study was the Minho River. All selected points are close to a source of anthropogenic impact (**Fig. 2**) The Minho River originates in Serra da Meira, in the province of Lugo (Spain) and drains into the Atlantic Ocean. It is approximately 300 km long, the last 70 km of which comprises its international section (the natural border between Portugal and Spain) Its hydrological basin has an area of 17 080 km^2, 95% of which is located in Spain, and only 5%, in Portugal (Sousa et al., 2008).

In the Portuguese portion of the river the population it is close to 25000 people in the river margin, distributed by the councils of Caminha, Vila Nova de Cerveira and Valença. There is a low degree of industrialization around this river, and the major contamination problem occurs due to domestic waist. In the last years there is an important industrial zone close to Vila Nova de Cerveira, however in the Spanish margin the industrial activity is intense (Alves, 1996). This increase in the industrialization of the area only reinforces the need to have monitoring programs in the region. With this study a gap in knowledge of the region could be fulfill.

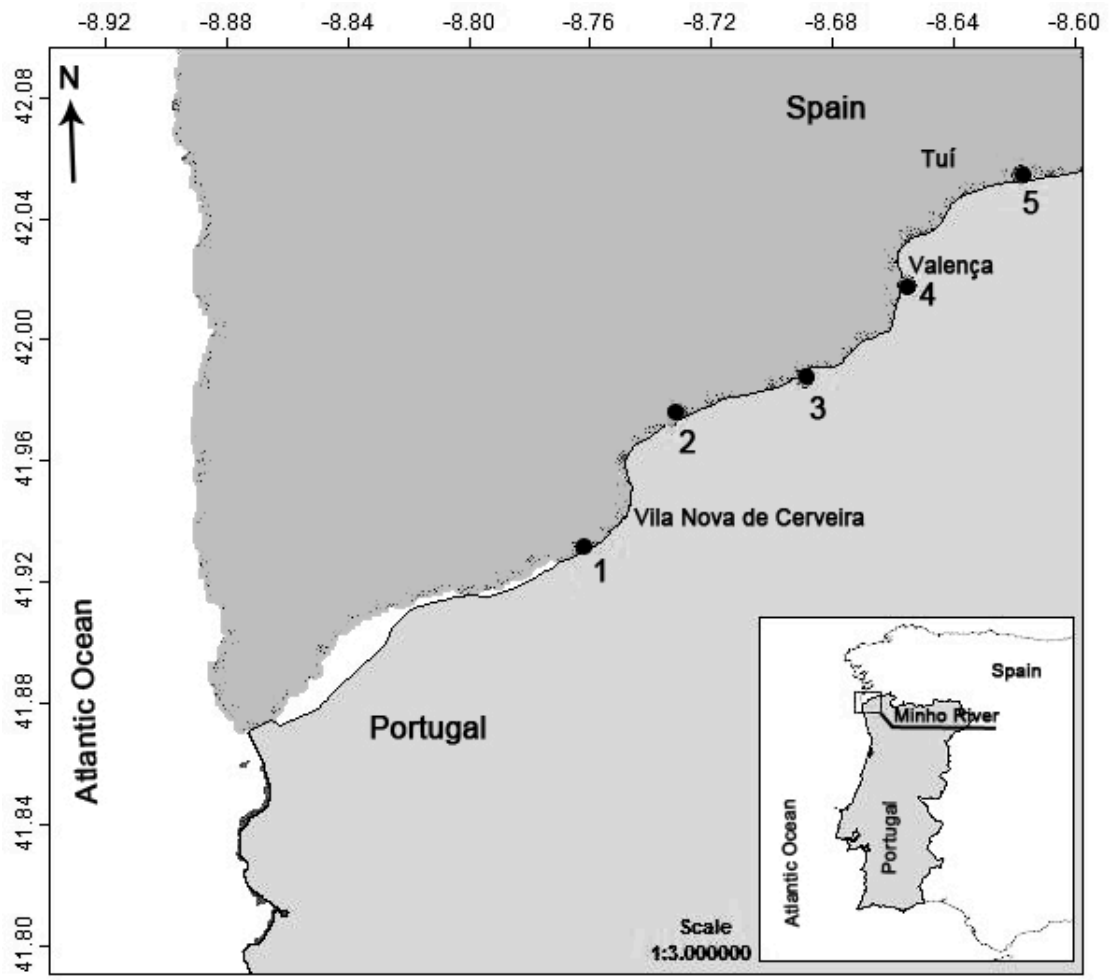


Figure 2. Map of Minho River showing the subsequent transplantation sites.

2.2 Organism selection and transplantation

2.2.1 Aveiro Estuary

The native species, *Mytilus galloprovincialis*, is present in the Aveiro Estuary at large communities in Point 1. Several organisms (n=600) were collected from this point (March of 2013) with high density of mussels and separated in 5 groups, each group were composed of 2 subgroups with 60 organisms. All groups were placed in a polypropylene mesh bag (80 mm) and left at the predefined points.

The sites were defined due to different types of contamination, varying from a touristic beach (Point 2), boat traffic and closeness to a major bridge (Point 3), marinas and Vouga River input (Point 4) to a control site (Point 1). The samples should be taken each 30 days, but this varied according to the weather forecast and vehicle availability. Samples were taken at 3 periods

during the 4 months experiment. Each time 10 organisms were collected to biomarker analysis purposes.

2.2.2 Minho River

The most common freshwater bivalve in Portugal is the invasive *Corbicula fluminea*; this non-indigenous species is present in several rivers, as the Minho. Since these organisms have the tendency to hide in the sediment and large densities were necessary to the transplant, a secondary location (N 40º 24´934", W 08º 45´073") was selected to collect the clams. Several organisms (n=1000) were collected in the spring (March of 2013) and separated in groups of 60 organisms, each group was placed in a polypropylene mesh bag (80 mm) and three bags were left in each point.

The points chosen in the Minho River started from Vila Nova de Cerveira, where the tides did not influence and the increase of salinity would not kill the organisms. All five points selected where close to a city, industrial park or touristic places. From the Point 1 all other points were upriver until the last point (5) was close to Tui, Spain. Each time 10 organisms were collected to biomarker analysis purposes. At some periods, due to the weather or other external conditions (fisherman, currents, mortalities, etc.), some bags were not retrieved.

2.3 Environmental variables

The physical-chemical variables, such as temperature, salinity, pH, conductivity and dissolved oxygen, were measured at all times and sample points, with exception of lost points where there was no organisms left to sample. Water for the quantification of chlorophyll-a and nutrients (silicate, phosphate, ammonia and nitrite) were collected in all sample points at all times, following the same pattern of the physical-chemical parameters. The samples for chlorophyll-a were filtered through Whatman GF/C glass filter (47 mm), the pigment was extracted with 90% acetone and a spectrophotometer analysis was performed according to described in Jeffrey & Humphrey (1975). The analysis of nutrients (silicate, phosphate, ammonium and nitrate) was performed with the filtered samples and was determined with the methods

described in UNEP/IOC/IAEA (1991). Data displayed in the supplementary material (**Tables 5 and 6**).

2.4 Body Condition Index

For each individual, length, width, heights of the shells were recorded and used to calculate condition indices of the individual bivalve. The condition index was calculated according to the following equations:

$$\text{Internal volume} = 3/4 * \text{length} * \text{width} * \text{height}$$

$$\text{Condition Index (CI)} = \text{Dry weight (g)} / \text{Internal Volume (cm}^3\text{)}$$

The dry weight was obtained after the dissection of organisms, the whole soft tissue from 10 individuals were pooled into a composed sample and dried at 45ºC for 48 hours and weighted.

2.5 Biomarker analysis

To analyze the effects of in situ transplantation in the digestive gland of *M. galloprovincialis* and *Corbicula fluminea* endpoints of neurotoxicity and oxidative stress were selected. The tissue was homogenized in phosphate buffer (0.01 M, pH 7.4), centrifuged for 20 minutes at 11500 rpm (Howcroft et al., 2011) and the post-mitochondrial supernatant (PMS) was used to determine the biomarkers, through spectrophotometrically measurement (Thermo Scientific Multiskan® Spectrum) in 96-well microplates.

2.5.1. Neurotoxicity enzymes

Three esterases enzymes were selected: acetylcholinesterase (AChe), butyrylcholinestrease (BChe) and propionylcholinesterase (PrChe). Ches were determined in the PMS, using 50 μL of sample and 250 μL of reaction buffer (30 mL K-Phosphate 0.1 M pH 7.2, 0.2 mL –choline substrate 0.075 M and 1 mL DTNB 10 mM). The activity was determined using an absorbance of 414 nm, following protocol described by Ellman, Courtney, Andres, & Featherstone (1961) and adapted to microplate by Guilhermino, Lopes,

Carvalho, & Soares (1996). Substrate analogues were acetylthiocholine iodide (ASCh), butyrylthiocholine iodide (BSCh) and propionylthiocholine (PrSCh).

2.5.2. Oxidative stress enzymes

Four endpoints were chosen to assess the oxidative stress produced through exposure do BP. Three were associate with the redox cycle (GST, CAT and GR) and one with the consequence of oxidative stress (LPO).

Glutathione S-Transferase (GST) activity was measured at 340 nm, following the methodology of Habig & Jakoby (1981) and adapted to microplate by Frasco & Guilhermino (2002). GST was determined in 100 μL of PMS and based on the conjugation product of GSH and CDNB. CAT was determined by the method of Clairborne (1985), and its activity was evaluated by kinetic measurement following the decrease in absorbance at 240 nm due to H_2O_2 decomposition.

Glutathione Reductase (GR) followed protocol described by Cribb, Leeder, & Spielberg (1989), where GR catalyzes the reaction of glutathione oxidade (GSSG) to glutathione (GSH) through oxidation of Nicotinamide adenine dinucleotide phosphate (NADPH), this activity was monitored at an absorbance of 340 nm. Levels of lipid peroxidation (LPO) were measured by method of Ohkawa, Ohishi, & Yagi (1979), were thiobarbituric acid (TBARS)-malondialdehyde (MDA) reactive species are generated. To 150 μL of PMS, 500 μL of 12% of trichloroacetic acid (TCA) in aqueous solution, 400 μL of 60mM Tris–HCl with DTPA 0,1 mM and 500 μL of 0.73% 2-thiobarbituric acid (TBA) were added and mixed well. The mixture was heated for one hour at 100ºC. The absorbance was read at 532 nm after removal of any particulate material by centrifugation.

Protein concentration was determined according to the Bradford (1976) method, using bovine serum albumin as standard. All Results are expressed in nmol min^{-1} mg $protein^{-1}$.

2.6 Statistical and data analysis

All the statistical analysis was analyzed using SPSS 21.0 software. Data were previously tested and did not pass the test for normality (Kolmogorov-Smirnov and Shapiro-Wilk tests) and homogeneity (Levene's test) A Kruskal-Wallis test with a Mann-Whitney U test as a post-hoc was performed to compare between stations and to verify differences among different periods per station. Pearson correlations were used to verify the influence of environmental factors on the physiology of bivalves and within physiological variables. Correlations were tested between physical-chemical parameters vs. biomarkers responses and body condition index. A Principal Component Analysis (PCA) with orthogonal rotation (varimax) was also performed to discriminate sites. Biomarkers (AChe, BChe, PrChe, GST, CAT, LPO, GR) and CI were the variables used in PCA. To ensure equal treatment during Pearson correlations and PCA analysis all variables were standardized, the method chosen was the Z score, with a mean of zero and a standard deviation of one.

3. Results

3.1 Condition Index

3.1.1 Aveiro Estuary

The condition index of mussels (**Fig. 3**) has a similar pattern to all stations, with a decrease after 30 days of experiment and an increase after 60 days of experiment. With exception of Point 1 at 90 days all points have significant differences when compared to the initial values (Mann-Whitney: $p<0.01$ for 30 days, $p<0.05$ for 60 and 90 days). Between points there is only distinction at 90 days of exposure where Points 1, 2 and 3 have significant differences to Point 4 (Mann-Whitney: $p=0.001$, 0.041 and 0.016 subsequently).

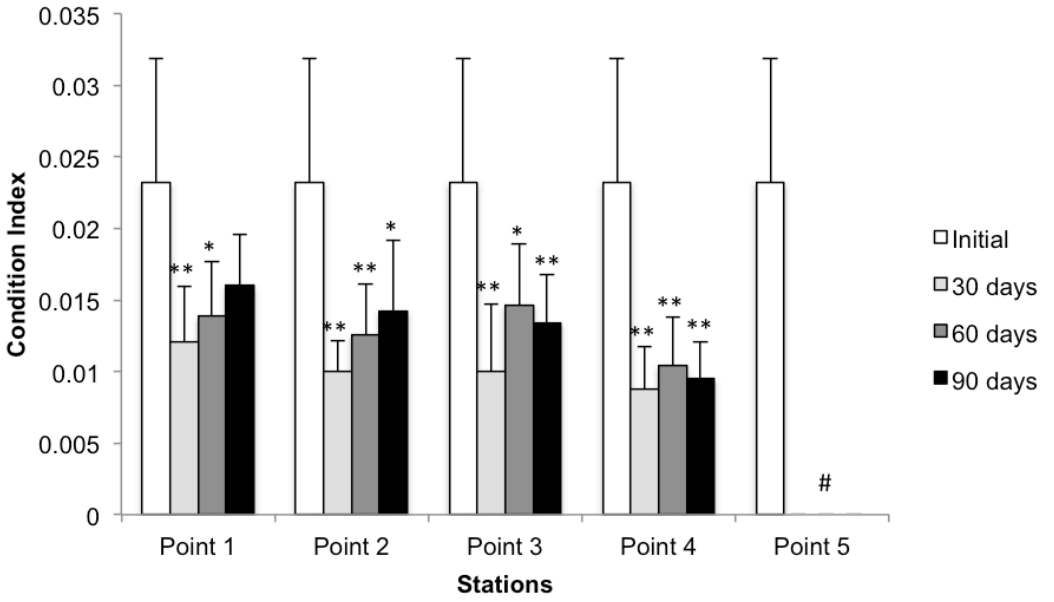


Figure 3. Condition index (C.I.) of mussels *Mytilus galloprovincialis* in different stations of Aveiro Estuary during 3 months. Data show the mean values and standard deviations (n=10). Statistical significance of the results is compared with the initial values (* < 0.05 and ** < 0.01) # Means that the point was lost.

3.1.2 Minho River

Condition index to *C. fluminea* (**Fig. 4**) follow a pattern where the values decreased after 30 days of experiment and remain lower than initial values (Kruskal-Wallis: $p<0.05$). Point 5 is the only one that demonstrates an improvement of CI values after 90 days of transplant. Among sites there is no significant differences, with one exception between points 4 and 5 at 90 days of experiment (Mann-Whitney: $p=0.013$).

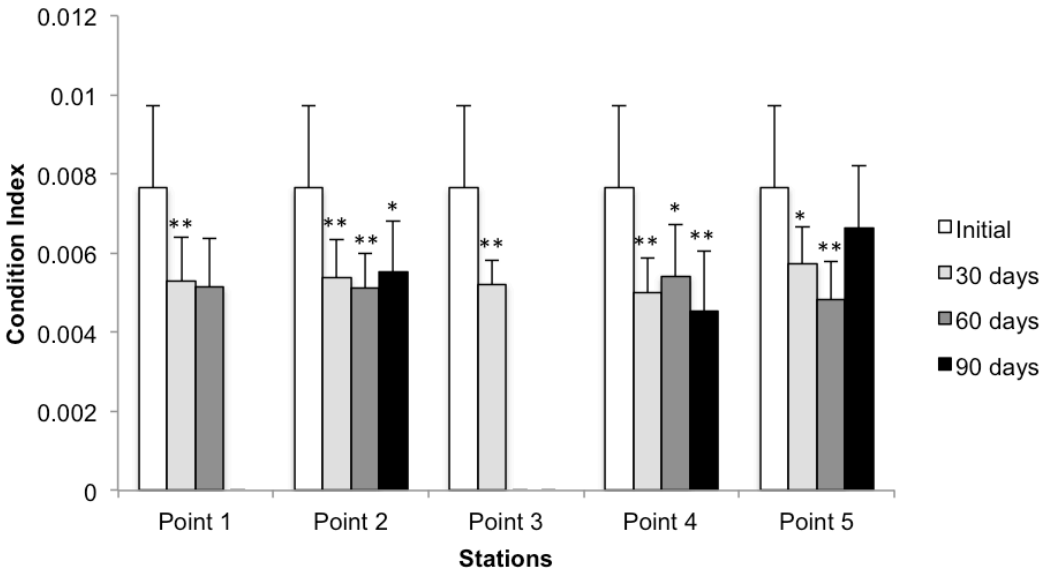


Figure 4. Condition index (C.I.) of clams *Corbicula fluminea* in different stations of Minho River during 3 months. Data show the mean values and standard deviations (n=10). Statistical significance of the results is compared with the initial values (* < 0.05 and ** < 0.01).

3.2 Neurotoxicity effects

3.2.1 Aveiro Estuary

The neurotoxicity enzymes (AChe, BChe and PrChe) demonstrated an induced activity over exposure time, especially at 90 days of experiment (**Fig. 5**). Enzymatic activity was expressed in different levels for each ChE following AChe > PrChe > BChe, consecutively by preference. AChe (**Fig. 5A**) remained unresponsive until 60 days of experiment, exception for Point 4 (Mann-Whitney: p=0.009); after 90 days of transplant a high induction is noticeable in Points 2, 3 and 4 (Mann-Whitney: p=0.001 for all data point). BChe (**Fig. 5B**) and Prche (**Fig. 5C**) show highly increased activity over time (Kruskal Wallis: p<0.01) with higher values after 90 days.

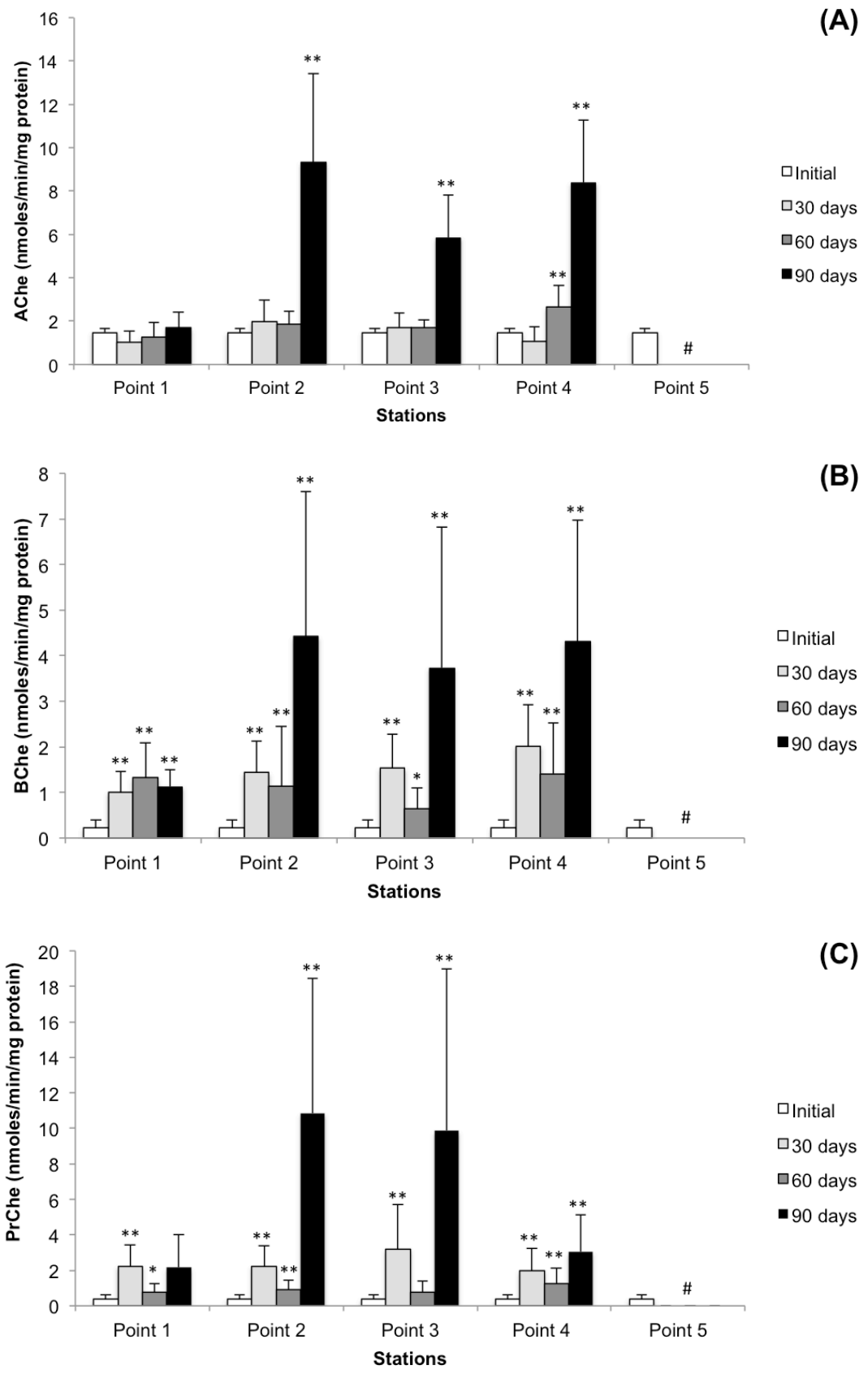
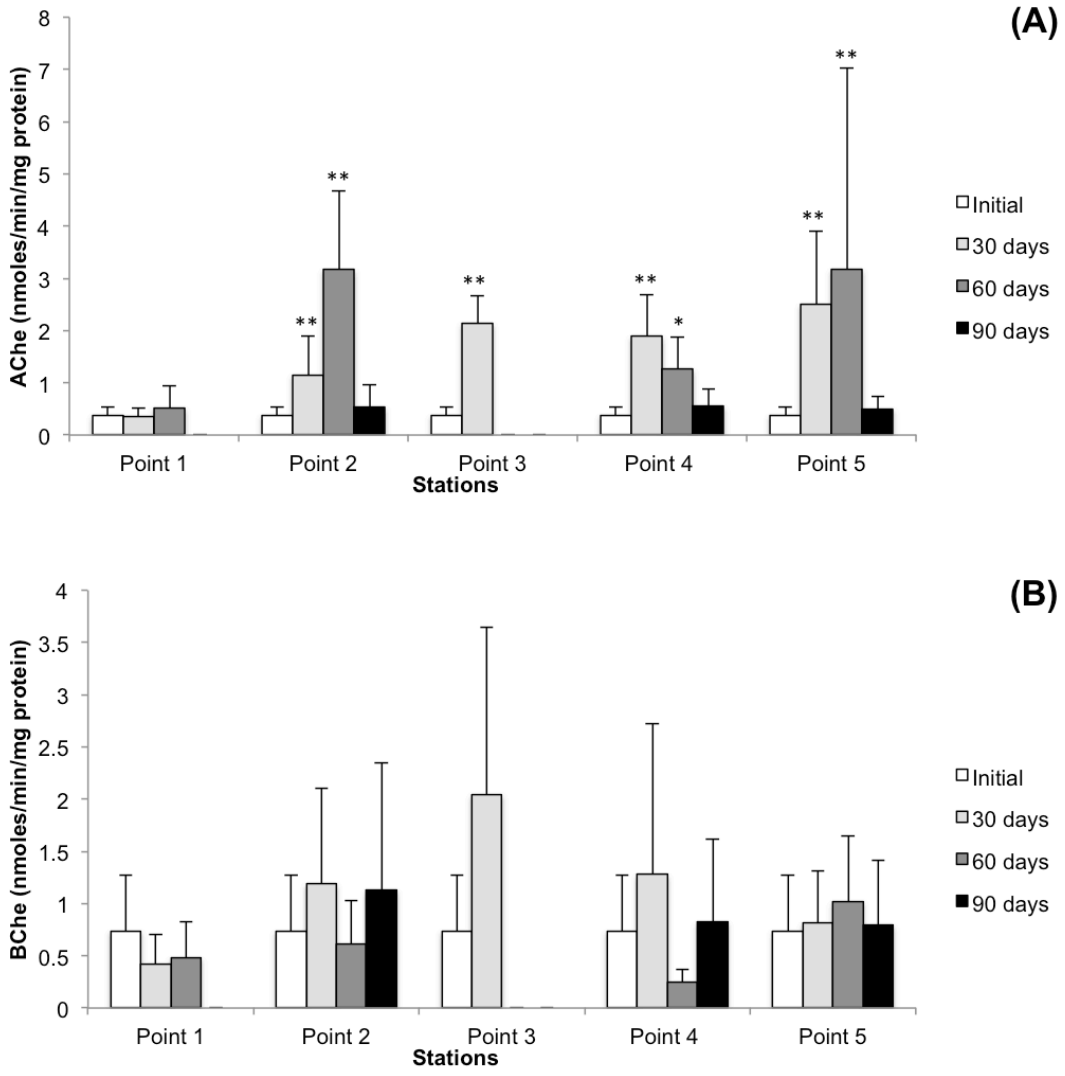


Figure 5. Biomarkers analyzed in digestive gland of *Mytilus galloprovincialis* in different stations from Aveiro Estuary. Data show the mean values and standard deviations (n=10) of (A) Acetylcholinesterase activity (AChe), (B) Butyrylcholinesterase (BChe), (C) Propionylcholinesterase (PrChe). Statistical

significance of the results is compared with the initial values (* < 0.05 and ** < 0.01)
Means that the point was lost.

3.2.1 Minho River

The neurotoxicity response of mussels retrieved from in situ experiment in Minho River (**Fig. 6**) show induced activity over time. Biomarkers were expressed in distinct levels for each ChE: AChe > BChe = PrChe, consecutively by enzymatic preference. AChe (**Fig. 6A**) had an increased activity over time (excepted in Point 1), with different trends for each point (Kruskal Wallis: p=0.000). BChe (**Fig. 6B**) and PrChe (**Fig. 6C**) demonstrate different trends over time but lacks significant differences (only exception to Point 1 at 30 days for PrChe; Mann-Whitney: p=0.010).



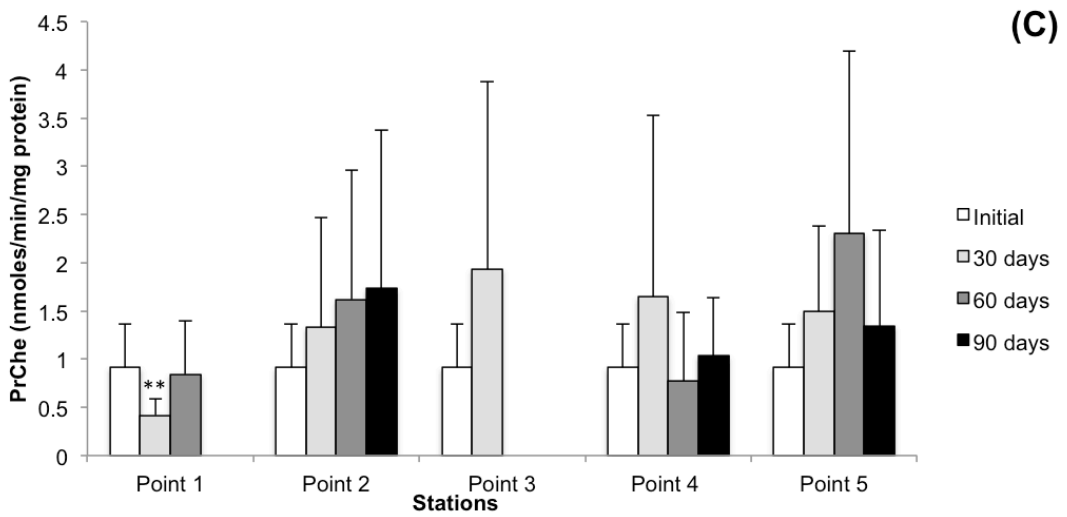
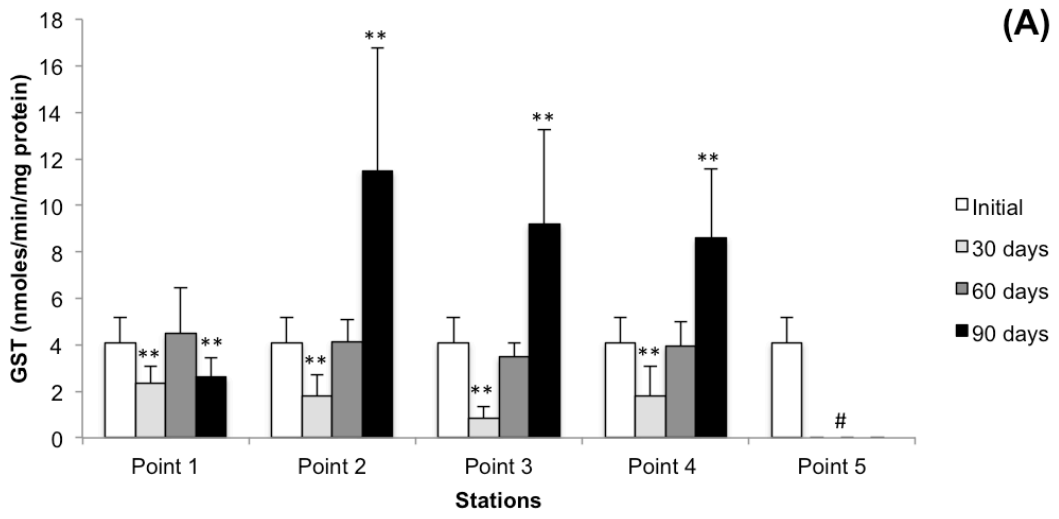


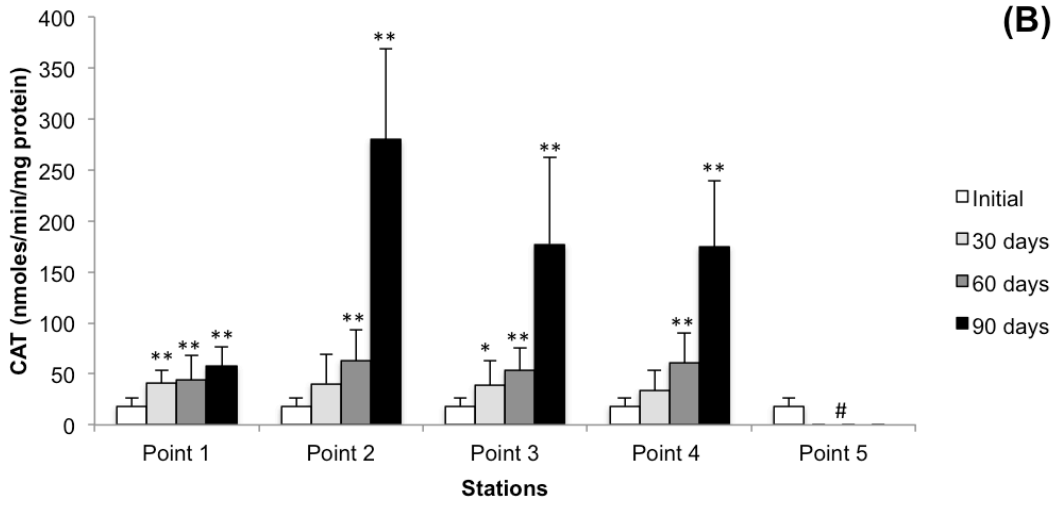
Figure 6. Biomarkers analyzed in digestive gland of *Corbicula fluminea* in different stations from Minho River. Data show the mean values and standard deviations (n=10) of A) Acetylcholinesterase activity (AChe), (B) Butyrylcholinesterase (BChe), (C) Propionylcholinesterase (PrChe). Statistical significance of the results is compared with the initial values (* < 0.05 and ** < 0.01).

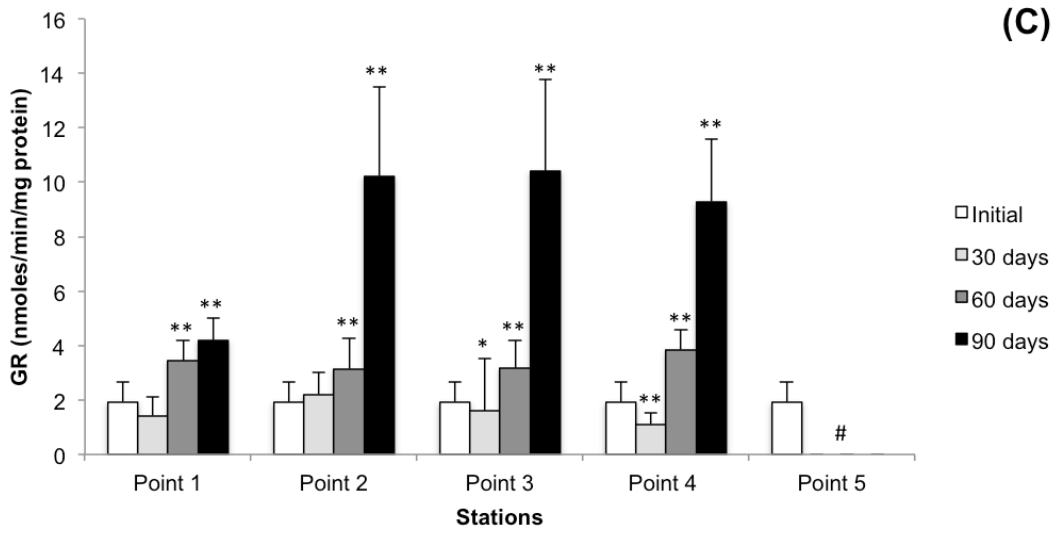
3.3. Oxidative stress effects

3.3.1 Aveiro Estuary

The oxidative-stress enzymes (GST, CAT, GR, and LPO) show increased enzymatic activity over time, especially after 90 days of experiment (**Fig. 7**). GST (**Fig. 7A**) exhibited inhibition at 30 days in all sites (Mann-Whitney: $p<0.01$) followed by induction at 90 days for Points 2, 3 and 4 (Mann-Whitney: $p=0.000$ for Point 2 and $p=0.004$ for Points 3 and 4). Point 1 differs significantly from all other points (Mann-Whitney: $p<0.01$). CAT (**Fig. 7B**) and GR activity (**Fig. 7C**) shows an exponential increase (Kruskal Wallis: $p<0.01$) with a peak at 90 days of transplant. LPO levels (**Fig. 7D**) rose after 30 days exposed (Mann-Whitney: $p<0.01$) and decreased over time (Points 3 and 4) or had a decreased followed by an increased (Points 1 and 2).


(A)
GST (nmoles/min/mg protein)
18
16
14
12
10
8
6
4
2
0
Point 1
Point 2
Point 3
Point 4
Point 5
Stations
Initial
30 days
60 days
90 days


(B)
CAT (nmoles/min/mg protein)
400
350
300
250
200
150
100
50
0
Point 1
Point 2
Point 3
Point 4
Point 5
Stations
Initial
30 days
60 days
90 days


(C)
GR (nmoles/min/mg protein)
16
14
12
10
8
6
4
2
0
Point 1
Point 2
Point 3
Point 4
Point 5
Stations
Initial
30 days
60 days
90 days

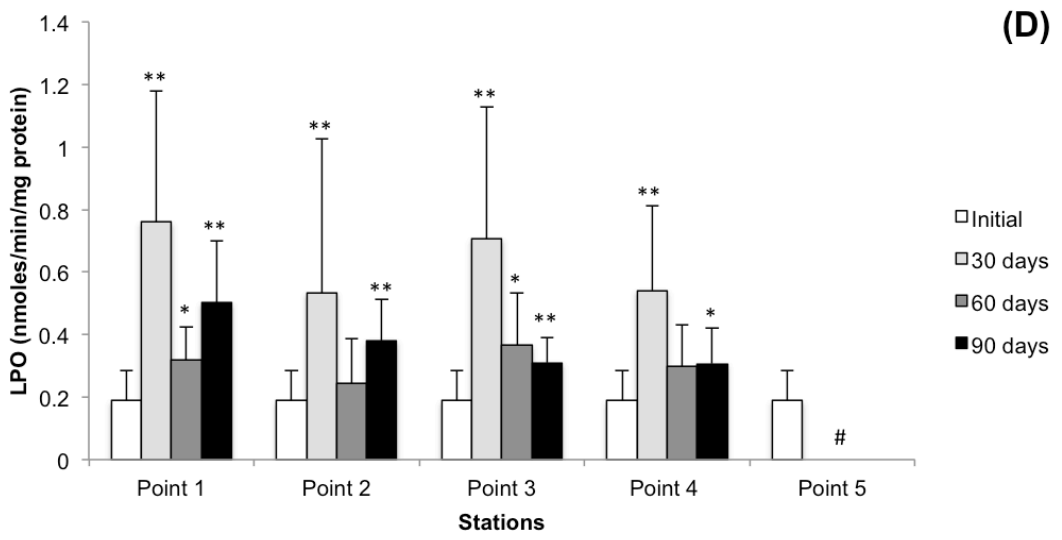
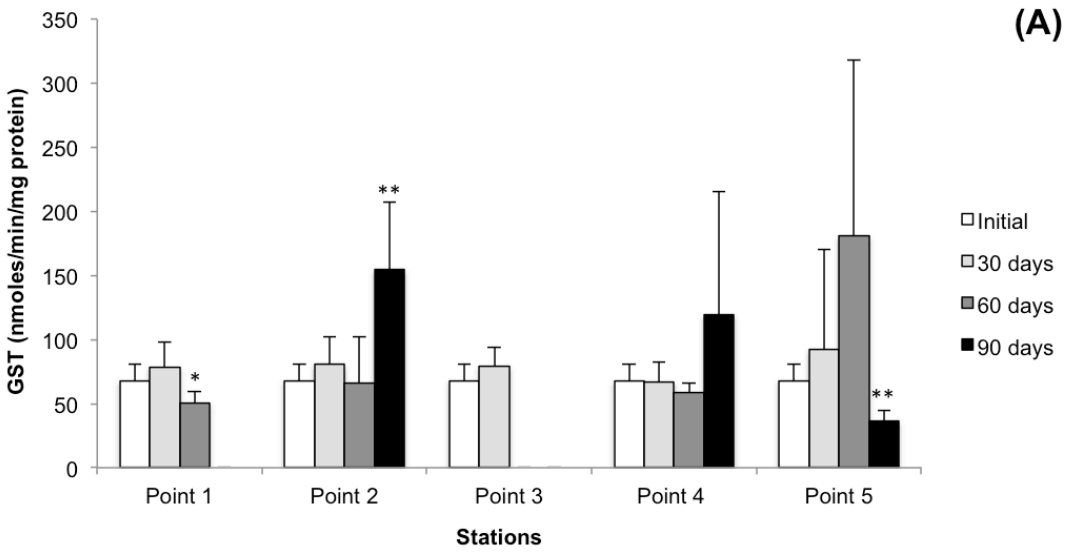
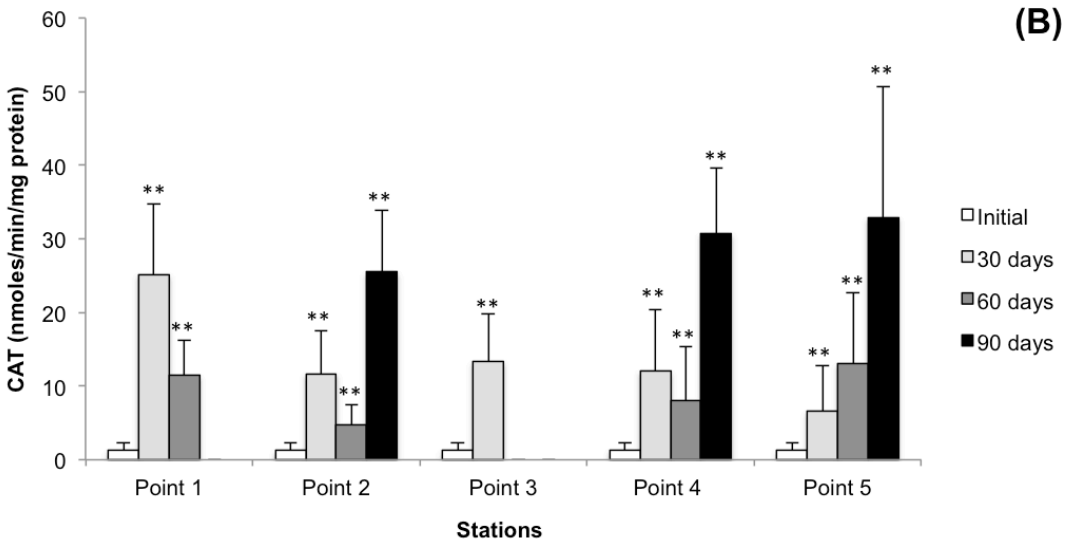


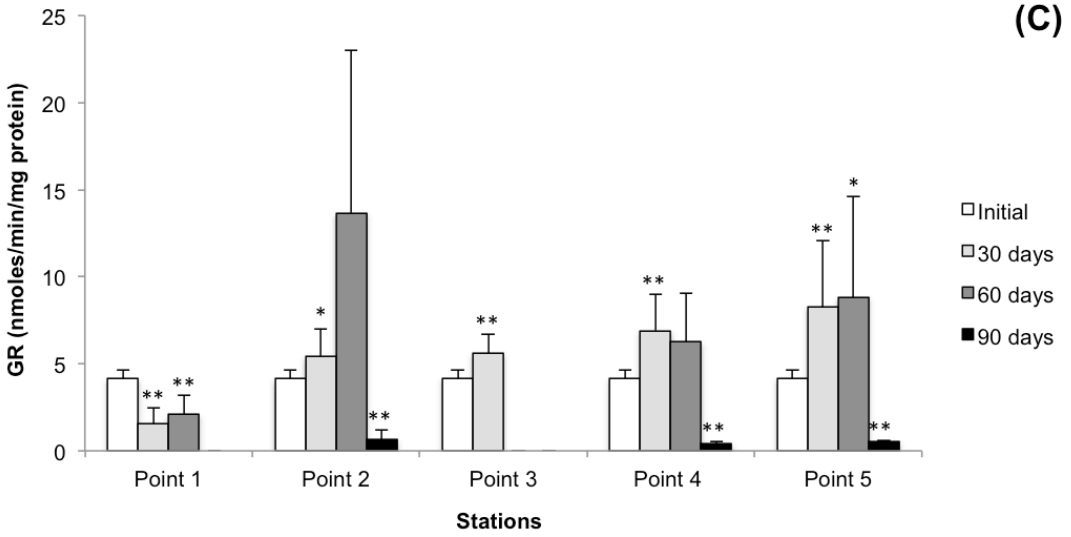
Figure 7. Biomarkers analyzed in digestive gland of *Mytilus galloprovincialis* in different stations from Aveiro Estuary. Data show the mean values and standard deviations (n=10) of (A) Glutathione S-Tranferase (GST), (B) Catalase (CAT), (C) Glutathione Reductase (GR), (D) Lipid Peroxidation (LPO). Statistical significance of the results is compared with the initial values (* < 0.05 and ** < 0.01) # Means that the point was lost.

3.3.2 Minho River

The oxidative stress-related enzymes (GST, CAT, GR and LPO) show different trends over exposure time (**Fig. 8**). GST (**Fig. 8A**) shows an inhibition in Point 1 at 60 days and in Point 5 at 90 days (Mann-Whitney: p=0.024 and p=0.000, subsequently), it also demonstrate induction in Point 2 at 90 days (Mann-Whitney: p=0.000). CAT (**Fig. 8B**) exhibited a crescent pattern over time, with peaks at 90 days (Mann-Whitney: p=0.000 for all points). GR activity (**Fig. 8C**) demonstrate an increased until 60 days (with exception of Point 1) followed by an extremely decreased at 90 days (Kruskal Wallis: p<0.01 for all time samples). Levels of LPO (**Fig. 8D**) decreased at Points 2 and 5 after 30 days of transplant (Mann-Whitney: p=0.010 and p=0.041). For all stress oxidative enzymes Points 1 and 5 differ the most amidst all studied sites.


(A)
GST (nmoles/min/mg protein)
350
300
250
200
150
100
50
0
Point 1
Point 2
Point 3
Point 4
Point 5
Stations
Initial
30 days
60 days
90 days


(B)
CAT (nmoles/min/mg protein)
60
50
40
30
20
10
0
Point 1
Point 2
Point 3
Point 4
Point 5
Stations
Initial
30 days
60 days
90 days


(C)
GR (nmoles/min/mg protein)
25
20
15
10
5
0
Point 1
Point 2
Point 3
Point 4
Point 5
Stations
Initial
30 days
60 days
90 days

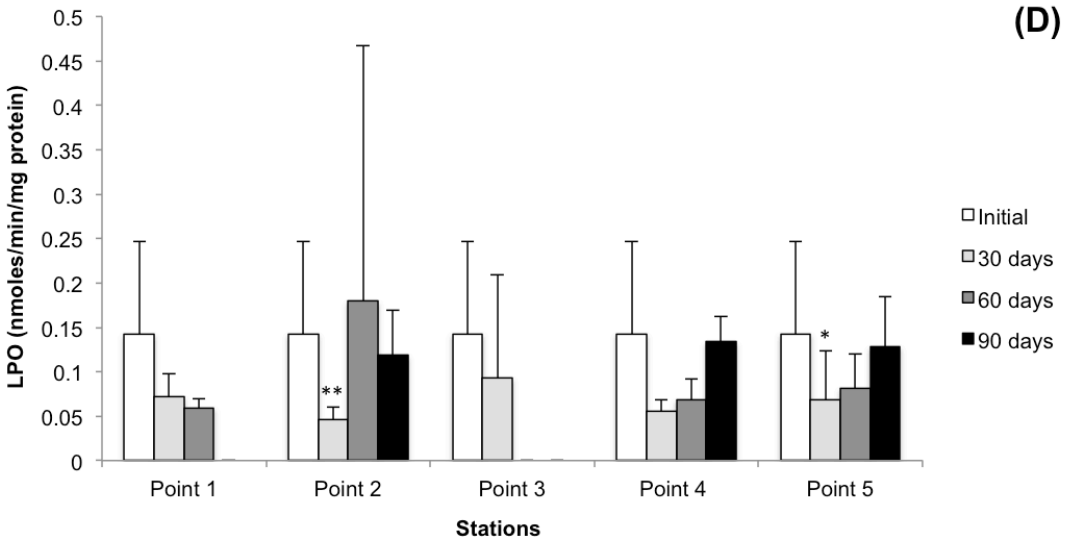


Figure 8. Biomarkers analyzed in digestive gland of *Corbicula fluminea* in different stations from Minho River. Data show the mean values and standard deviations (n=10) of (A) Glutathione S-Tranferase (GST), (B) Catalase (CAT), (C) Glutathione Reductase (GR), (D) Lipid Peroxidation (LPO). . Statistical significance of the results is compared with the initial values (* < 0.05 and ** < 0.01).

3.4 Relationship between physiological responses and environmental variables

3.4.1 Aveiro Estuary

The Pearson correlation for the selected endpoints (biomarkers, condition index and physic-chemical parameters) demonstrates a high influence of temperature and levels of dissolved oxygen in the organism response (**Table 3**). The most influenced variable by the abiotic factors was the condition index.

Table 1. Pearson correlation coefficient between biomarker responses in digestive gland of *Mytilus galloprovincialis*, condition index (CI) and environmental variables in Aveiro Estuary.

	AChe	BChe	PrChe	GST	CAT	GR	LPO	CI
Temperature	**0.753***	**0.853****	**0.745****	**0.491***	**0.764****	**0.753****	0.414	**-0.572***
Conductivity	-0.111	-0.213	-0.154	-0.023	-0.159	-0.134	-0.235	0.336
pH	-0.150	-0.243	-0.018	-0.262	-0.186	-0.242	0.172	0.180
Salinity	0.322	**0.526***	0.358	0.189	0.440	0.443	0.416	**-0.719****
DO (mg/L)	**-0.607****	-0.388	-0.368	**-0.610****	**-0.499***	**-0.539***	0.376	-0.338
Chlorophyll a	-0.227	-0.366	-0.278	-0.043	-0.233	-0.164	-0.381	0.389
Silicate	-0.167	-0.393	-0.232	-0.049	-0.292	-0.287	-0.424	**0.703****
Phosphate	0.070	-0.151	-0.021	0.197	-0.002	-0.031	-0.401	**0.555***
Nitrite	-0.141	-0.341	-0.362	-0.022	-0.306	-0.257	**-0.495***	**0.621****
Ammonia	0.472	0.329	0.064	0.379	0.285	0.315	-0.242	0.168

Data were pooled across sites. Values and asterisks in bold indicate significant relationships (*p<0.05, **p<0.01).

3.4.2 Minho River

Pearson correlation (biomarkers, CI and physico-chemical parameters) demonstrates an influence of abiotic factors in the response of selected endpoints (**Table 2**). The levels of dissolved oxygen affected several endpoints, including AChe, PrChe, CAT and CI. The most influenced biomarkers were AChe, CAT and the condition index. There are no other patterns worth mention.

Table 2. Pearson correlation coefficient between biomarker responses in digestive gland of *Corbicula fluminea*, condition index (C.I.) and environmental variables in Minho River.

	AChe	BChe	PrChe	GST	CAT	GR	LPO	CI
Temperature	0.031	0.025	0.296	0.315	**0.788***	-0.326	-0.085	**-0.590***
Conductivity	-0.032	-0.136	-0.042	-0.072	0.264	-0.205	-0.393	**0.487***
pH	0.329	**0.613****	0.337	-0.091	-0.030	0.223	**-0.603***	-0.260
DO (mg/L)	**-0.526***	-0.358	**-0.635****	0.411	**-0.513***	-0.106	0.471	**0.803****
Chlorophyll a	-0.379	-0.200	-0.285	-0.026	0.007	-0.312	0.368	0.213
Silicate	**0.649****	-0.356	0.287	0.081	-0.208	**0.644****	-0.210	-0.390
Phosphate	-0.271	-0.242	-0.364	-0.261	-0.296	-0.060	0.319	0.389
Nitrite	**-0.486***	0.028	-0.267	0.069	0.230	**-0.525***	0.364	0.104
Ammonia	-0.321	-0.376	-0.319	-0.099	**0.502***	-0.434	-0.162	-0.306

Data wee pooled across sites. Values and asterisks in bold indicate significant relationships (*p<0.05, **p<0.01).

3.4 Relationship between endpoints

3.4.1 Aveiro Estuary

The PCA (**Fig. 9 and 10**) indicate that 2 Principal Components could explain the set of variables (biomarkers and CI) and is responsible for 92.05% of total variance (**Table 3**). While almost all endpoints have an influence in the first component: CAT, GR, Ache, BChe, PrChe, GST weight positively and CI negatively and with 68.58% of original variance; the second principal component explained 23.47% of the variance: positive values were associated with GST and CI and the negative value with LPO.

Table 3. PCA: Component loadings of the variables for the two principal components in Aveiro Estuary.

Variables	*Component 1*	*Component 2*
Eigen values	*5.486*	*1.878*
% of variance	*68.574*	*23.473*
CAT	*0.989*	*-*
GR	*0.965*	*-*
AChe	*0.964*	*-*
BChe	*0.954*	*-*
PrChe	*0.894*	*-*
GST	*0.885*	*0.443*
LPO	*-*	*-0.943*
CI	*-0.392*	*0.832*

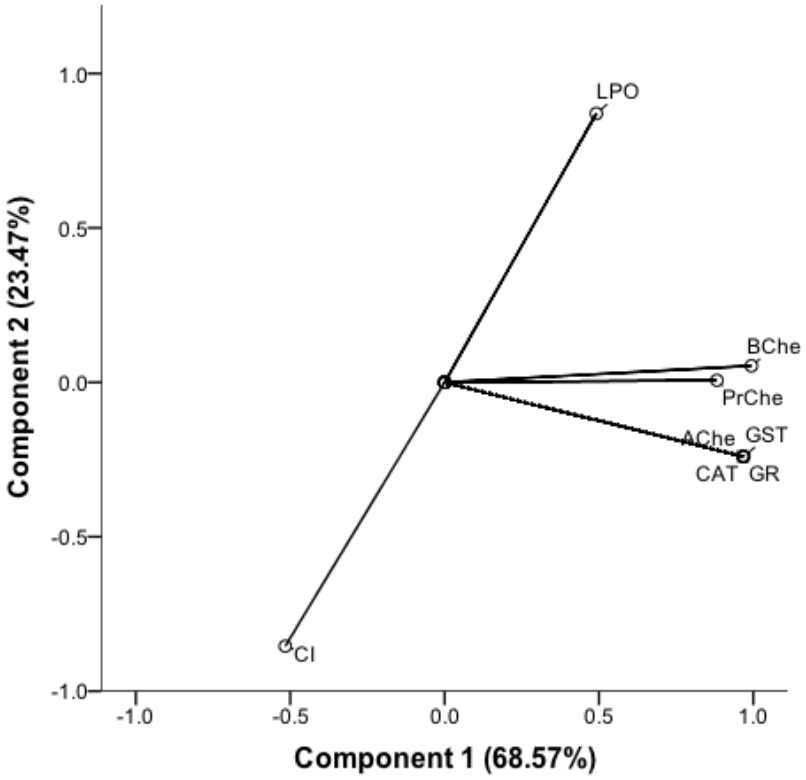


Figure 9. Plot of variable vectors for the two dominant components produced by biomarkers (Ache, GST, CAT, LPO, GR) and condition index (C.I.) of Aveiro Estuary.

Plot of scores for the two principal components of different sites and time exposure (**Fig. 10**) showed a clear pattern. The response of organisms tends to be more time-dependent; the three groups formed consist basically of different sites clustered by the same sample time (30, 60 or 90 days). The

initial values are all grouped together and stood apart of other groups. At 30 days there is a strong influence of several endpoints (AChe, BChe, PrChe, GST, CAT, GR) (**Fig. 9**), at 60 days all endpoints have an influence in the organisms response, oppositely after 90 days of transplant there is a clear impact in the outcome from LPO levels only.

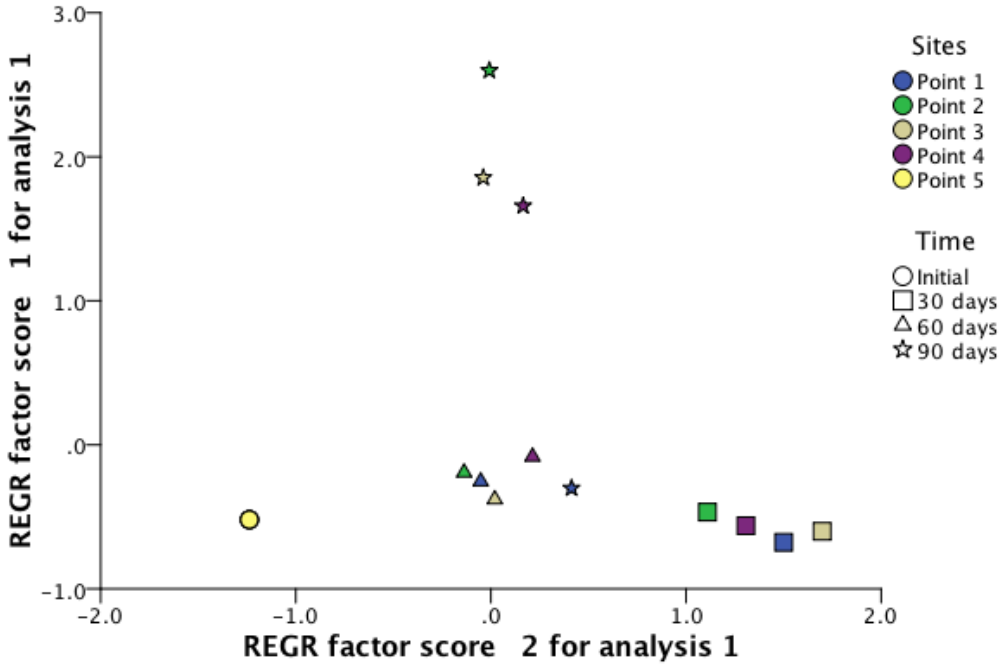


Figure 10. The distribution diagram of the different groups of sites during different experimental periods as a function of the two principal component axis. Principal component loading and total variance associated with each axis are provided in Table 3.

3.4.2 Minho River

PCA results (**Fig. 11 and 12**) indicate that selected data (biomarkers and CI) could be explained by 3 principal components that accounted for 80.36% of total variance (**Table 4**). The Principal Component 1 is responsible for 41.06% of the variance and has positive influences of PrChe, Ache, GST, BChe, GR and negative of CI and LPO. Principal Component 2 was accounted for 24.83% of the total variance; AChe, CI, GR and LPO influenced positively while CAT negatively. The Principal Component 3 explained 14.47% of variance and was impacted only positively by PrChe, CI, GST, BChe and LPO.

Table 4. PCA: Component loadings of the variables for the two principal components in Minho River.

Variables	*Component 1*	*Component 2*	*Component 3*
Eigen values	*3.285*	*1.986*	*1.157*
% of variance	*41.059*	*24.828*	*14.469*
PrChe	*0.899*	*-*	*0.344*
AChe	*0.872*	*0.401*	*-*
CI	*-0.723*	*0.439*	*0.417*
GST	*0.606*	*-*	*0.350*
BChe	*0.590*	*-*	*0.509*
CAT	*-*	*-0.880*	*-*
GR	*0.549*	*0.778*	*-*
LPO	*-0.372*	*0.409*	*0.617*

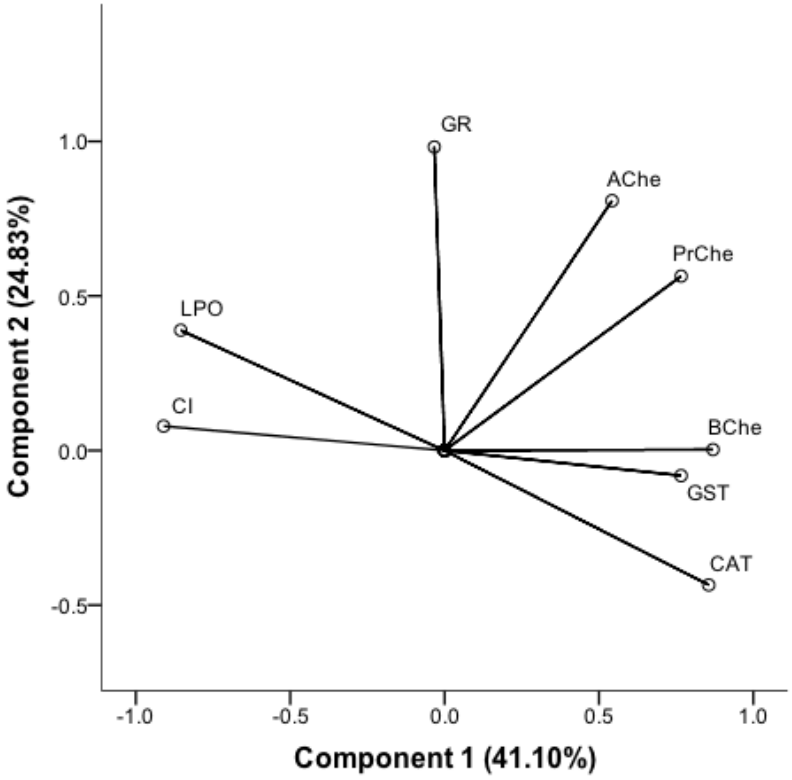


Figure 11. Plot of variable vectors for the two dominant components produced by biomarkers (Ache, GST, CAT, LPO, GR) and condition index (C.I.) of Minho River.

The plot of scores for the two principal components separated four groups of sites and periods (**Fig. 12**). Initial values plus Points 2, 4 and 5 at 30 days formed one group; this outcome is utterly influenced by AChe, PrChe, BChe, GST and CAT (**Fig. 11**). Point 1 at 30 and 60 days and Point 4 at 90

days grouped together, and had no major endpoints influencing this response. Point 3 at 30 days and Point 5 at 60 days cluster together and are influenced by GR and AChe. The remaining points at 90 days gather closely and had major influences of LPO and CI. Point 2 at 60 days is isolated and is not associated with any of the groups formed.

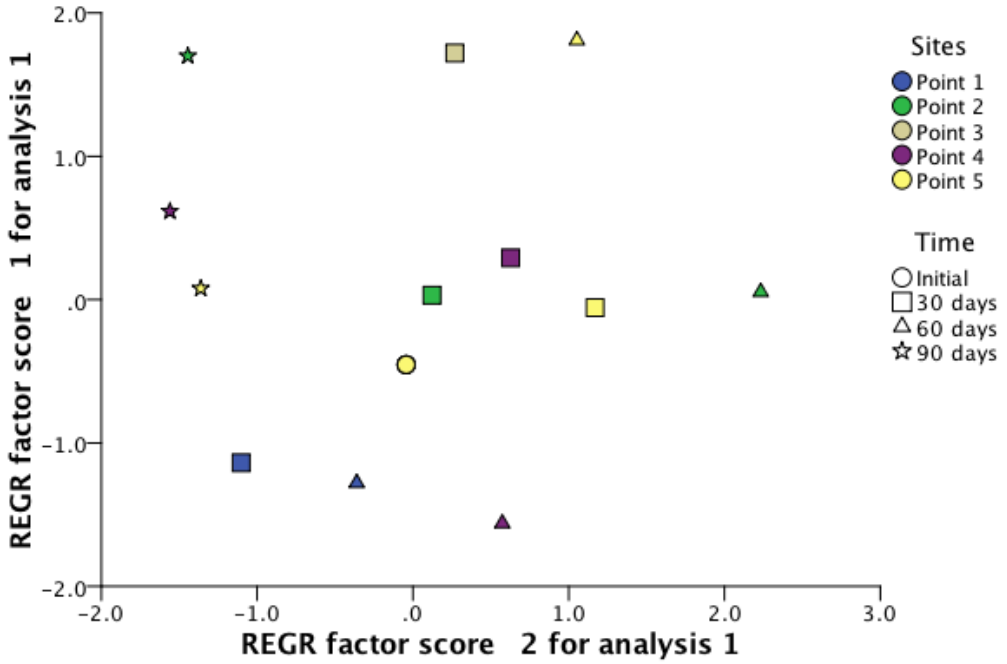


Figure 12. The distribution diagram of the different groups of sites during different experimental periods as a function of the two principal component axis. Principal component loading and total variance associated with each axis are provided in Table 4.

4. Discussion

In this study we aimed to compare the influence of different types of contamination (rivers inflow-related pesticides and anthropogenic impact-related hydrocarbons) in locations of known impact in the sublethal response of bivalves during a certain amount of time. We found a variety of response in different environments. Neurotoxicity results displayed a great induction of all Ches forms in Aveiro while in the Minho River this trend is only pronounced for AChe. The oxidative stress response in Aveiro displayed high increased in all enzymatic activities (GST, CAT and GR) leading to damage in the health state of bivalves, reflected in high levels of LPO. The same pattern is not observable in the river compartment, where there is only peak activity for CAT and GR and no damage to the organism's physiology was reflected, leading

to believe that *C. fluminea* has a great detoxification system to deal with xenobiotics present in the location.

Comparing physiological responses from *C. fluminea* and *M. galloprovincialis* in different environments: estuary (Aveiro Estuary) and river (Minho River), apparently the overall response in both places is more time-related then site specific. The influence of some endpoints to this response at specific time samples is also similar, leading to believe that the seasonal influences had a greater significance in this results then the presence of contaminants.

4.1 Aveiro Estuary

Regarding neurotoxicity results, *M. galloprovincialis* showed a substrate preference for ASCh followed by PrSCh and BSCh, corroborated by several authors (Brown et al., 2004; Lau and Wong, 2003; Talesa et al., 2001; Yaqin, 2010). The timely induction of AChe activity in summer (90 days) was supported by other studies with the same trend (Binelli et al., 2005; Cravo et al., 2012; Palais et al., 2012). Neurotoxic pesticides (cabamate and organophsphorus) are known to employ an inhibition in AChe (Führer et al., 2012; Fulton and Key, 2001; Gagné et al., 2010; Viarengo et al., 2007). However, some authors (Galloway et al., 2002; Rickwood and Galloway, 2004) pointed out that enzymatic activity is only significantly decreased in the presence of high concentrations of pesticides, what could be a possible explanation for our results. AChe peaked only at 90 days, while other ChEs (BChe and PrChe) had also an increased activity in the other sample times (30 and 60 days). This induced activity could be a mechanism to prevent the overload of the detoxification pathway; furthermore, pseudocholinesterases (BChe and PrChe) play a protective role towards AChe, scavenging anti-cholinergic compounds (Masson and Lockridge, 2010; Salles et al., 2006; Sanchez-Hernandez, 2007).

Lionetto et al. (2003) observed that in control or less polluted locations the ChE activity increase, this could be also a possible explanation for the higher values; since Point 1 is closer to the channel connected with the sea and have a high traffic of boats and ships. The transplant to other points could

lead to a detoxification of the organisms, hence a higher enzymatic activity. It is well know that Che activity is affected by abiotic factors (Burgeot et al., 2010; Dellali et al., 2001; Frasco et al., 2010; Leiniö and Lehtonen, 2005), mainly by temperature. In our study all ChEs enzymes are correlated with temperature, suggesting an important influence in the neurotoxicity response of mussels over time.

Temperature and other abiotic variables as dissolved oxygen can affect the antioxidant response of bivalves; Sheehan & Power (1999) already postulated that antioxidant defenses are under extensive seasonal control. All the major antioxidant enzymes (GST, CAT and GR) are induced up to 15x at 90 days of transplant, this could be a seasonal effect, since these enzymes are strongly correlated to temperature and dissolved oxygen.

It is very well established that the induction of GST often is associated with the presence of xenobiotics (Armknecht et al., 1998; Rocher et al., 2006; Willett et al., 1997) and its induction can be considered a signal of physiological adaptation to organic contamination (Maranho et al., 2012). In our study the high levels of GST could be linked to the presence of contaminants, in Aveiro Estuary there is a historical contamination of metals (Pereira et al., 2009; Sousa et al., 2007) and organic compounds (Antunes and Gil, 2004) what could be associated with the results, since the constant inflow of tides re-suspend contaminants and could be responsible for this outcome.

According to some field studies (Akcha et al., 2000; Cheung et al., 2004; Cossu et al., 1997; Cotou et al., 2013; Livingstone, 2001; Pampanin et al., 2005; Regoli and Principato, 1995). CAT enzymatic activity could increase, decrease or remain the same. In our study an exponential increased occurred and this could be due to contaminant-mediated induction of ROS as a protective mechanism against oxidative stress (Cossu et al., 1997). GR is involved in the antioxidant defense through the maintenance of GSH/GSSG homeostasis under oxidative stress conditions (Winston and Di Giulio, 1991). With our results is possible to relate the activity of GR to GST and notice that at high inductions of GST a high increased of GR followed. This trend was not valid for some sample points, probably because GST inhibition occurred due to the lack of GSH substrate to follow the detoxification pathway.

Lipid peroxidation (LPO) occurs as a consequence of environmental stressors, such as light or exposure to xenobiotic compounds (Halliwell and Guteridge, 1985). Lipid peroxidation can be observed when antioxidant and detoxifying systems are deficient and not active enough to neutralize the active intermediates produced by xenobiotic and their metabolites (Vasseur and Cossu-Leguille, 2003); a possible explanation of what happened in our study.

The antioxidant defenses are not able to protect the organism to oxy damages; corroborated by LPO levels that increased when the other enzymes are lower. Over time it is possible to notice that the damage caused by oxyradicals decreased since the enzymatic activity increased leading to a better protection of the physiology of the organism. This could be an adaptation of its surroundings, however this conclusion should be taken carefully, since LPO is not correlated with any other enzyme, but it is correlated with CI. CI decreased over time, mainly due to the weight of organisms, this could be a shift after spawning period, also observed in other field study (Cravo et al., 2012). Another explanation for the damage through stress oxidative could be linked to the health state of organism; CI is related to BChe, all Ches usually have a neurotransmission role in the organism. An explanation for the reduced values of CI could be related to a higher activity of BChe, leading to a stress and a close state of organism, affecting its weight and the health state.

4.2 Minho River

The results for the Minho River show a discrepant pattern for the biomarkers and there is no similar response in the endpoints activity. Regarding neurotoxicity results, *C. fluminea* showed a substrate preference for ASCh followed equally by PrSCh and BSCh, Ramos, Gonçalves, Antunes, & Nunes (2012) also displayed the same substrate preference for this species. AChe was the only neurotoxic enzyme to be affected in this experiment; several studies demonstrate that cholinesterase activities in this species are quite insensitive to pesticide exposure and varied across seasons (Binelli et al., 2007; Damásio et al., 2010; Mora et al., 1999), explaining our results. According to Dimitriadis, Gougoula, Anestis, Pörtner, & Michaelidis

(2012) AChe is considered highly sensitive to biotic and abiotic changes, in our results this enzyme was correlated to several physico-chemical parameters.

GSTs can be induced by diverse contaminants, namely PAHs, PCBs, furans, phenobarbital compounds and others (Cunha et al., 2005; Hartl et al., 2007; van der Oost et al., 2003). However GST can be also inhibited in response to some pesticides (Robillard et al., 2003), mostly because time of exposure or type of xenobiotics can differentiate the expression of some GST's isoforms (Boutet et al., 2004). Our study reflects decreased values at some points and increased at others. Several authors reported increased, decreased or unchanged GST activities by exposure to several xenobiotics (Akcha et al., 2000; Cotou et al., 2013; Gowland et al., 2002; Regoli et al., 2004; Robillard et al., 2003). The induction could be associated to the presence of persistent organic contaminants and/or metals; a study of Mil-Homens et al. (2013) pointed out the presence of these pollutants due to nautical activities, urban and industrial activities with a high exposition of contaminant sediment resulted of dredging activities in the River.

Induction of CAT could also be a response to pollution, since the antioxidant defense system can be induced by contaminant-mediated production of ROS as a protective mechanism against oxidative stress (Cossu et al., 1997). Other studies related this induction to the presence of contaminants (Cotou et al., 2013; Richardson et al., 2008; Vlahogianni et al., 2007) other then seasonal variations. However, as mentioned before Sheehan & Power (1999) postulated that antioxidant defenses are under extensive seasonal control; with CAT being correlated to several abiotic factors it is hard to conclude the main mechanism behind this enzymatic response.

GR role is to maintain the intracellular concentration of GSH with consumption of NADPH. GR induction is often associated as biomarker of oxidative stress (Stegeman et al., 1992; Zhang et al., 2004), a possible explanation for our results. GST and GR are linked through GSH recycling, however they are not correlated in our results and maybe the individual responses of each are not associated. Indifferently, LPO responses lack significance differences over time, implying that oxidative stress enzymes

efficiently counteracted the intracellular ROS formation: preventing permanent damages to clams' physiology.

4.3 General Comparison

In overall there is high differences among environments, but comparing PCAs it is possible to make some observations, regardless the environment (River or Estuary) the response of organisms is mainly time-related. In both compartments the response at 30 days is more afflicted by ChEs and oxidative stress (GST, CAT and GR) while the final sampling time (90 days) have a high influence of LPO levels. The abiotic factors influencing the responses are entirely different on both transplants, due to their different hydrodynamics, but in the end this does not affected the general response. This considerations should be taken carefully, since this could be a process of adaptation to the surroundings and not an overall response of how the genre behave when impacted by xenobiotics *in situ*. Transplants taking into account a higher amount of time should be considered as well the insertion of genomics and proteomics studies, and analysis of contaminants to be able to check if this is response is related to the presence of specific contaminant or it is seasonal, and if this behavior is a genetically adaptation to a chronically polluted site or not.

5. Conclusion

Response of organisms in overall tends to be more time-dependent, this is more clear in Aveiro than in Minho, this could be due to the tides influence, that mostly distributed contaminants over all sample points, in other hand this is not something affecting the response in Minho river. We should also take into consideration that we began the experiment in the spring and finished in summer, what could be the most important factors for the behavior here experienced by these organisms. Especially in Aveiro, were temperature is correlated with all endpoints, what does not happen in Minho River. Further studies should take place with a higher battery of endpoints to better understand the physiology of organism in these places and to have a better difference among sites, which could elucidate the relationship between local pollution and enzymatic response.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

Authors acknowledge Fundação para Ciência e Tecnologia (FCT - Portugal) by the financial support through the grant SFRH/BPD/62561/2009 (Jessica C.L. Ladewig) and the Biology Department of Aveiro University.

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SUPPLEMENTARY MATERIAL

Table 5. Environmental parameters measured monthly from Point 1 to 4 in Aveiro Estuary between November 2011 and March 2012. T- temperature, C – conductivity, S - salinity, DO – dissolved oxygen, Chl-a – chlorophyll-a, Sil – silicate, Phos – phosphate, Nitr – nitrite, Amm - ammonium.

Points	Month	T (ºC)	C (μS/cm)	pH	S	DO (mg/L)	Chl-a (mg/cm^3)	Sil (μmol/L)	Phos (μmol/L)	Nitr (μmol/L)	Amm (μmol/L)
1	Mar 13	15.9	36.7	8.1	22.9	8.51	11.65	1940	20.66	255	586
	May 13	17.8	47.6	8.2	30.8	10.33	3.06	643	10.57	158	299
	Jun 13	15.7	52.2	6.93	33.8	9.8	16.17	196	16.5	194	176
	Jul 13	21.3	51	7.98	33.2	7.08	3.88	592	14.89	133	466
2	Mar 13	15.5	12.76	8.15	7.2	8.01	15.90	4809	77.37	687	325
	May 13	19	33	8.13	20.5	9.53	6.54	2246	20.66	231	360
	Jun 13	15.3	51.4	7.9	33.3	9.4	4.75	250	12.01	152	288
	Jul 13	22	44.7	7.83	28.8	5.84	5.77	1549	67.28	168	502
3	Mar 13	15.9	31.5	8.23	19.8	8.48	29.83	2584	61.51	513	562
	May 13	19.1	44.2	8.15	28.5	8.73	2.71	1130	15.85	166	234
	Jun 13	16.3	48.3	7.98	31	8.94	48.94	605	14.09	101	168
	Jul 13	21.4	50.2	7.99	32.7	7.5	12.82	838	14.89	122	302
4	Mar 13	14.7	20.6	8	12.1	7.02	7.78	2353	11.69	308	615
	May 13	18.1	41	7.89	24.9	9.06	10.22	1178	16.33	276	452
	Jun 13	18.2	47.7	8.01	30.7	9.8	7.94	509	11.53	190	161
	Jul 13	22.5	51.4	7.73	33.6	5.72	5.38	887	23.54	382	987

Table 6. Environmental parameters measured monthly from Point 1 to 5 in Minho River between March 2013 and July 2013. T- temperature, C – conductivity, DO – dissolved oxygen, Chl-a – chlorophyll-a, Sil – silicate, Phos – phosphate, Nitr – nitrate, Amm - ammonium.

Points	Month	T (ºC)	C (µS/cm)	pH	DO (mg/L)	Chl-a (mg/cm^3)	Sil (µmol/L)	Phos (µmol/L)	Nitr (µmol/L)	Amm (µmol/L)
1	Mar 13	10.8	73	7.55	10.9	4.38	1692	66.32	160.12	235.68
	Apr 13	13.5	82.4	7.95	9.8	0.82	1399	16.1	92.35	330.53
	May 13	20.4	161.3	7.38	8.86	7.76	3532	21.14	129.86	408.95
	Jul 13	-	-	-	-	-	-	-	-	-
2	Mar 13	10.5	71.2	7.56	11.2	2.31	2278	23.54	155.07	188.36
	Apr 13	13.2	83	9.52	9	3.17	1901	15.49	115.9	188.67
	May 13	17.2	85.5	7.19	8.86	3.39	3885	20.66	94.56	191.92
	Jul 13	26.1	90.6	7.53	7.35	2.98	1648	8.65	141.21	482.25
3	Mar 13	11.3	69.4	7.35	10.7	18.99	1590	16.09	123.55	94.62
	Apr 13	14	84	9.3	8	1.05	1201	14.89	122.3	183.93
	May 13	22.7	93.3	7.27	8	32.70	3970	13.09	94.56	172.38
	Jul 13	-	-	-	-	-	-	-	-	-
4	Mar 13	10.3	70.7	7.44	11.3	3.94	1919	13.29	139.94	142.38
	Apr 13	15.6	86.8	10.04	9.2	0.24	2745	4.48	112.21	131.5
	May 13	16.7	84.3	7.15	8.65	1.96	3413	18.74	97.08	704.38
	Jul 13	22.6	93.6	6.98	9.44	13.71	1883	20.50	263.51	415.83
5	Mar 13	10	70.6	7.34	11.3	4.71	1838	14.89	139.94	101.29
	Apr 13	13.1	78.4	9.49	8.5	0.90	3551	16.5	114.73	116.1
	May 13	16.3	83.7	7.23	-	2.80	3762	7.68	76.59	63.97
	Jul 13	22.8	84.7	7.49	9.25	4.45	2104	14.89	119.78	410.8

CHAPTER

4

BIOMARKERS AS A SUBLETHAL TOOL FOR SHORT AND LONG-TERM EXPOSURE OF BENZO(A)PYRENE IN MUSSELS (*MYTILUS GALLOPROVINCIALIS*)

Biomarkers as a sublethal tool for short and long-term exposure of Benzo[a]pyrene in mussels (*Mytilus galloprovincialis*)

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This chapter was submitted as an original article to:

Aquatic Toxicology

Abstract

Bivalves are often used to assess the effects of several contaminants, mostly due to its ability to accumulate xenobiotics. Several studies have been trying to understand the underlying sublethal effects caused by polycyclic aromatic hydrocarbons (PAHs) in bivalves. Based on prior knowledge from we selected five concentrations of Benzo[a]pyrene, BaP, (10, 20, 35, 60 and 100 μg/L) plus a control and a solvent control to assess the long-term response of mussels in selected ecological and biochemical endpoints. Regarding the assessment of neurotransmission function, acetylcholinesterase (AChe) and propionylcholinesterase (PrChe) activities were inhibit during short-term exposure, in addition PrChe, was only affected during long-term exposure. Butyrylcholinestrease (BChe) show no significant effect caused by BaP. The use of more than one cholinesterase showed great potential to a better interpretation of neurotoxicity caused by PAHs. Enzymatic activity of GST and GR decreased over time, while CAT increased, this trend was associated with antioxidant system and detoxification pathway of BaP and metabolites. LPO displayed no significant effect, which is indicative that the protection defenses of *M. galloprovincialis* against oxidative stress were not supressed and therefore this organism in controlled conditions (laboratory conditions) has the potential to coexistent with high concentrations of BaP. We evaluated the contribution of all independently measured variables by principal components analysis (PCA), and we verified that time of exposure has a more significative weight in mussels response when compared with a gradient of concentrations. To a better understanding of physiological responses, studies of metabolites and/or genomics should be incorporated in future researches.

Keywords: *Mytilus galloprovincialis*, bivalves, Benzo[a]pyrene, cholinesterases, oxidative stress, antioxidant system.

1. Introduction

Mussels, particularly *Mytilus* spp., stand as an ideal biomonitoring organism with high ecological importance, wide geographical distribution, and are sessile in nature, thereby preventing them from moving away from contaminant exposure. As filter feeders, mussels tend to bioaccumulate contaminants in a greater extent than the surrounding media (Cajaraville et al., 2000); even though, these organisms respond significantly to contaminant exposure, maybe explaining the wide variety of biological effects. Several techniques can measure these adverse biological effects – from molecular to the whole organism responses – what could represent early warning signals of environmental disturbance (Hylland et al., 2006).

The use of biological response to stress (or biomarkers) in sentinel species became a major topic in environmental quality evaluation and risk assessment (Bayne, 1985; Depledge, 1994; Van der Oost et al., 2003; Verlecar et al., 2008). Metabolism and energetics-related biomarkers allow an association between biochemical responses to contaminants and the changes in higher biological organization levels (Depledge et al., 1995).

Benzo[a]pyrene (BaP) is a polycyclic aromatic compound (PAH) mainly associated to fossil fuels. In the last decades, the PAHs-related pollution has been aggravating in the marine environments and they arise from different sources: highly marine traffic, oils spills and offshore oil platforms (Volodkovich and Belyaeva, 1992; Xiu et al., 2014). BaP can activate the metabolic system once inside the cells, producing Phase I metabolites (i.e. epoxides, quinones and diolepoxides) through biotransformation; then, to avoid higher toxicity, Phase II enzymes conjugate this metabolites facilitating the elimination of xenobiotic (Hellou et al., 2012). Glutathione-S-Transferase (GST) is the most studied enzyme from Phase II; Cappello et al. (2013), Pan et al. (2005), Wang et al. (2011) linked the activity of GST to the presence of PAHs.

According to Livingstone (2001), some PAH metabolites can generate reactive oxygen species (ROS) via redox cycling; In fact, several studies (Liu et al., 2014; Maria and Bebianno, 2011; Pan et al., 2009, 2008; Wang et al., 2011; Xiu et al., 2014) associated the effects of PAHs to oxidative stress in bivalves. ROS-scavenging enzymes (i.e. catalase and glutathione reductase)

must stop the propagation of lipid peroxidation (LPO) and permanent cellular damage.

Cholinesterases (Ches) are fulcral for the transmission of nervous influx, being the specific target for most nervous agents and insecticides (Barata et al., 2004; Basack et al., 1998; Escartín and Porte, 1997; Galloway et al., 2002; Jebali et al., 2013; Vioque-Fernández et al., 2007); thus, to understand the mode of action of ChEs we should study the sensitivity of specific inhibitors, since it may differ across species (Bianco et al., 2014). There are no studies relating the responses of different Cholinesterases to PAHs. Yang et al. (2002) showed the potential for ChEs activity on onset apoptosis in nerve cells, thus to better understand the effects associated with neurodegeneration in mussels, studies associating PAHs with this enzymes are a very important fist step.

For this study, we aimed to assess the sublethal response of different endpoints in *Mytilus galloprovincialis* exposed to benzo(a)pyrene in a short and long-term experiment, taking into account neurotoxicity and ROS-scavenging enzymes. We hoped to better understand the detoxification pathway and to contribute to validate the use of the selected biomarkers as a panel for the assessment of oxidative stress impairment in the context of persistent organic pollutants (POP's).

2.Materials and Methods

2.1 Chemicals and preparation of solutions

Benzo(a)pyrene (96% purity) was purchased from Alfa Aesar (Germany). Stock solution of benzo(a)pyrene (200 mg/L) was carefully prepared with a ratio of 1:9 acetone to artificial seawater, it was kept refrigerated and protected from light. Test solutions were prepared immediately before the beginning of the test by successive dilution of the stock.

2.2 Organism selection and assay

Mytilus galloprovincialis was selected to assess the effects of benzo(a)pyrene in some endpoints. The organisms were collected from Aveiro Estuary in April of 2013. Around 300 organisms were collected in the

estuary and fouling organisms were removed; subsequently they were acclimatized under laboratory conditions for 7 days priori the beginning of the experiment. Mussels were treated under natural light and keep under starvation for the first 4 days of bioassay, in the chronic exposure organisms were feed every other day with homogenized TetraMin fish food (TetraWerke, Melle, Germany) and, water was renewed each 4 days. Five different concentrations were chosen for benzo(a)pyrene (10, 20, 35, 60 and 100 μg/L), one control and solvent control using acetone. A first set of mussels's digestive gland tissue was dissected out and was considered the initial values for the experiment, in the subsequent time samples (1, 2, 3, 4, 10 and 21 days) 7 individual fractions per exposure were dissected and stored at -80ºC until enzymatic activity analysis.

2.2.1 Body Condition Index

For each individual, length, width, heights of the shells were recorded and used to calculate condition indices of the individual bivalve. The condition index was calculated according to the following equations:

$$\text{Internal volume} = 3/4 * \text{length} * \text{width} * \text{height}$$

$$\text{Condition Index (CI)} = \text{Dry weight (g)} / \text{Internal Volume (cm}^3\text{)}$$

The dry weight was obtained after the dissection of organisms, the whole soft tissue from 10 individuals were dried at 45ºC for 48 hours and weighted.

2.3 Physico-chemical variables

During the experiment, several physico-chemical parameters were monitored, as temperature, conductivity, dissolved oxygen, pH and salinity with a multi-parameter sensor. Levels of nitrite and ammonia were also monitored to ensure no interference in the effects of the contaminant and they follow tolerance levels pointed out by (Epifanio and Srna, 1975). The analysis

of nitrite and ammonia were performed with the methods described in (UNEP/IOC/IAEA, 1991).

2.4 Biomarkers analysis

To analyze the effects of benzo(a)pyrene (BaP) in the digestive gland of *M. galloprovincialis* endpoints of neurotoxicity and oxidative stress were selected. The tissue was homogenized in phosphate buffer (0.01 M, pH 7.4), centrifuged for 20 minutes at 10000 g (Howcroft et al., 2011) and the post-mitochondrial supernatant (PMS) was used to determine the biomarkers.

2.4.1 Neurotoxicity enzymes

Three esterases enzymes were selected: acetylcholinesterase (AChe), butyrylcholinestrease (BChe) and propionylcholinesterase (PrChe). ChEs were determined in the PMS, using 50 μL of sample and 250 μL of reaction buffer (30 mL K-Phosphate 0.1 M pH 7.2, 0.2 mL –choline substrate 0.075 M and 1 mL DTNB 10 mM). The activity was determined using an absorbance of 414 nm, following protocol described by Ellman et al. (1961) and adapted to microplate by Guilhermino et al. (1996). Substrate analogues were acetylthiocholine iodide (ASCh), butyrylthiocholine iodide (BSCh) and propionylthiocholine (PrSCh).

2.4.2 Oxidative Stress enzyme

Four endpoints were chosen to assess the oxidative stress produced through exposure do BP. Three were associate with the redox cycle (GST, CAT and GR) and one with the consequence of oxidative stress (LPO).

Glutathione S-Transferase (GST) activity was measured at 340 nm, following the methodology of Habig and Jakoby (1981) and adapted to microplate by Frasco and Guilhermino (2002). GST was determined in 100 μL of PMS and based on the conjugation product of GSH and CDNB. CAT was determined by the method of Clairborne (1985), and its activity was evaluated by kinetic measurement following the decrease in absorbance at 240 nm due to H_2O_2 decomposition.

Glutathione Reductase (GR) followed protocol described by Cribb et al. (1989), where GR catalyzes the reaction of glutathione oxidade (GSSG) to glutathione (GSH) through oxidation of Nicotinamide adenine dinucleotide phosphate (NADPH), this activity was monitored at an absorbance of 340 nm. Levels of lipid peroxidation (LPO) were measured by method of Ohkawa et al. (1979), were thiobarbituric acid (TBARS)-malondialdehyde (MDA) reactive species are generated. To 150 μL of PMS, 500 μL of 12% of trichloroacetic acid (TCA) in aqueous solution, 400 μL of 60mM Tris–HCl with DTPA 0,1 mM and 500 μL of 0.73% 2-thiobarbituric acid (TBA) were added and mixed well. The mixture was heated for one hour at 100ºC. The absorbance was read at 532 nm after removal of any particulate material by centrifugation.

Protein concentration was determined according to the Bradford (1976) method, using bovine serum albumin as standard. All Results are expressed in nmol min^{-1} mg $protein^{-1}$.

2.5 Statistical analysis

Statistical analyses were performed with SPSS 21.0 software. Comparisons were made between concentration series and time of exposure using a Kruskal-Wallis test and Mann-Whitney U test as a post-hoc. A priori all data were tested for normality (Kolmogorov-Smirnov and Shapiro-Wilk tests) and homogeneity (Levene's test) and did not pass the tests. Pearson correlations were used to verify the relationships among the different biomarkers and the physical-chemical parameters. A Principal Component Analysis (PCA) with orthogonal rotation (varimax) was also performed to simplify the correlation structures and to compare the physiological response of *Mytilus galloprovincialis* to Benzo(a)pyrene. Biomarkers (AChe, BChe, PrChe, GST, CAT, GR and LPO) and CI (condition index) values were the variables used in PCA. To ensure equal treatment during Pearson correlations and PCA analysis all variables were standardized, the method chosen was the Z-score, with a mean of zero and a standard deviation of one. REGR factor score; explain analysis principle and which variables were taken in account

3.Results

3.1 Body Condition Index

The condition index (C.I.) of mussels exposed to BaP show a clear decay along time (**Fig. 1**). At day 2, the C.I. of PAHs-exposed mussels (10 and 60 μg/L) show significant differences against control (Mann-Whitney: p=0.018).

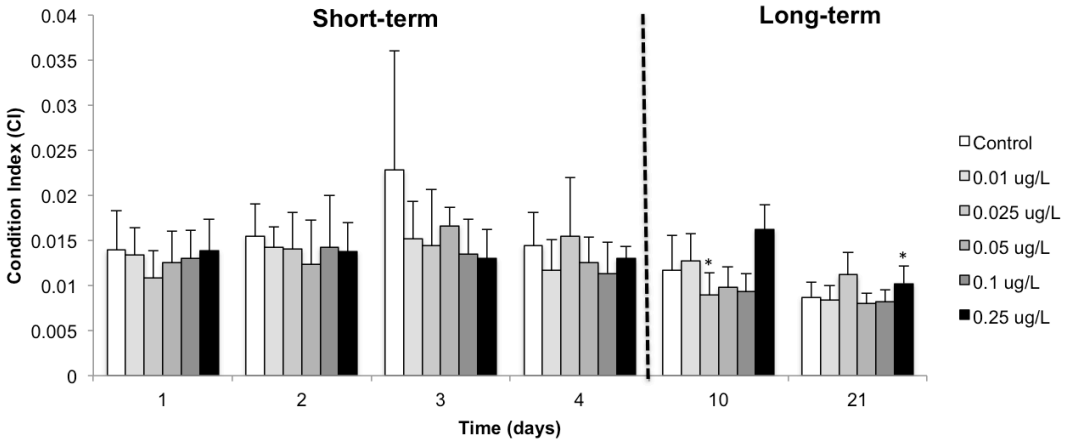


Figure 1. Condition Index (C.I.) of *Mytilus galloprovincialis* exposed to different concentrations to benzo(a)pyrene. Data show the mean values and standard deviations (n=7). Statistical significance of the results is compared with the control values (* < 0.05).

3.2 Neurotoxicity effects

The neurotoxicity enzymes (Ache, BChe and PrChe) demonstrated different trends along the experiment (**Fig. 2**.). Enzymatic activity was expressed in different levels for each ChE following AChe > PrChe > BChe. AChe (**Fig. 2A**) was inhibited at day 2 and 4 (Mann-Whitney: p=0.013 for day 2; p=0.05 for day 4). BChe (**Fig. 2B**) was induced but lacks significant differences. PrChe (**Fig. 2C**), the most affected biomarker, was inhibited at day 3, 4 and 21 (Mann-Whitney: $p<0.05$ and $p<0.01$); being the only neurotoxicity enzyme affected in the long-term exposure.

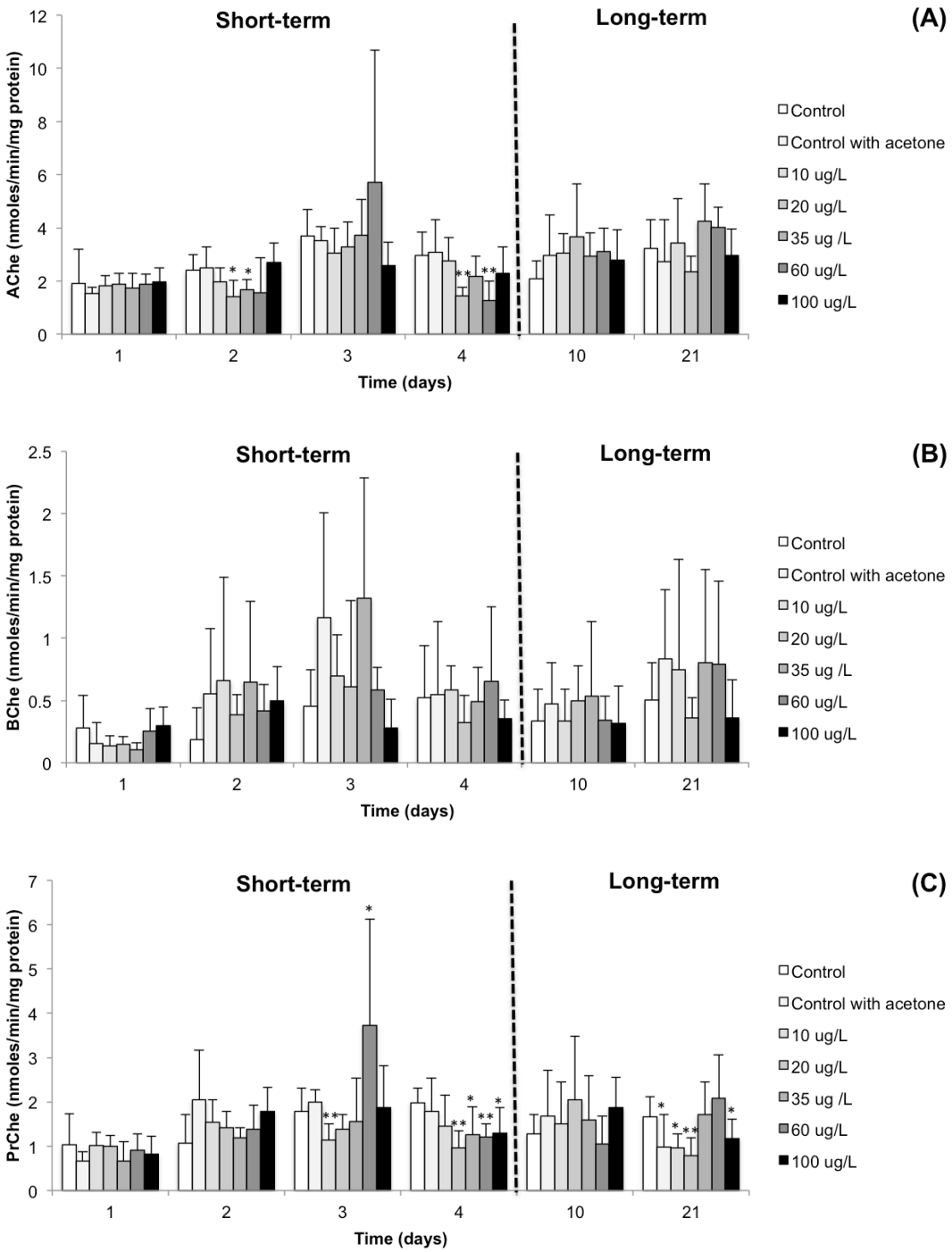
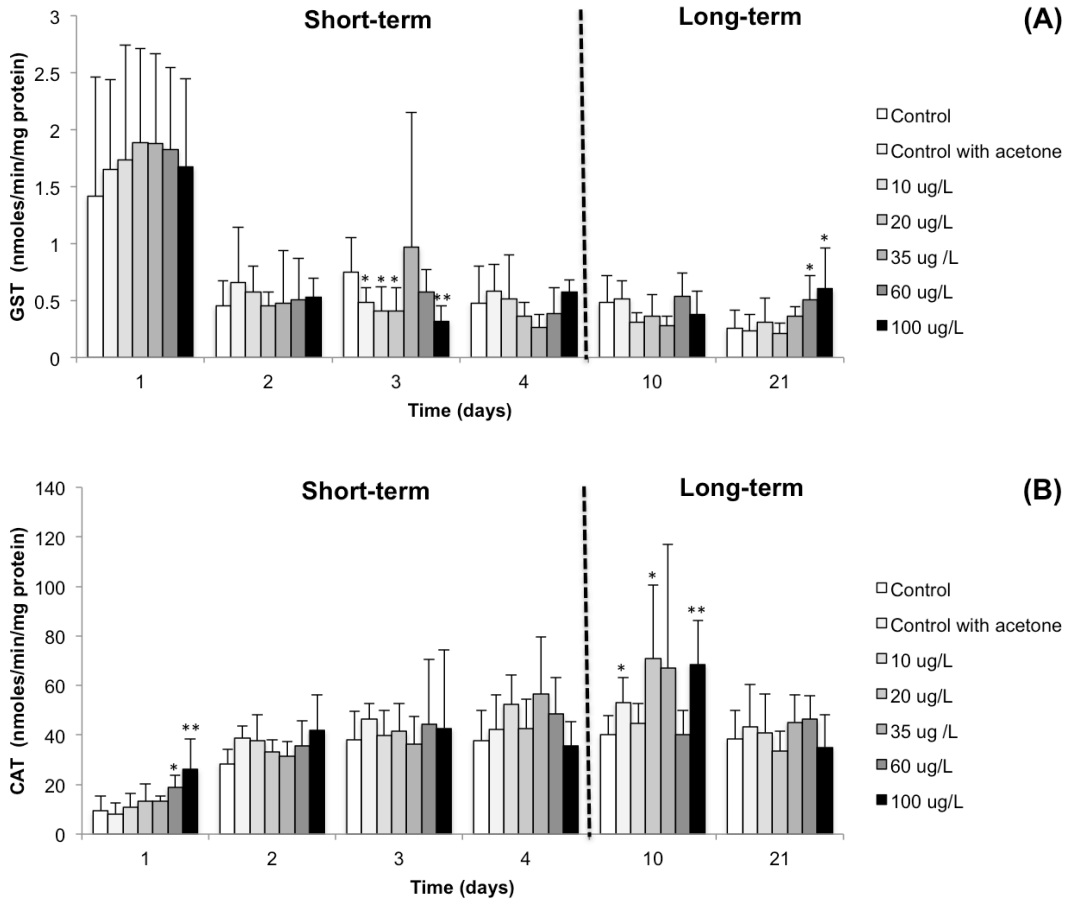


Figure 2. Biomarkers of neurotoxicity analyzed in digestive gland of *Mytilus galloprovincialis* exposed to different concentrations of benzo(a)pyrene. Data show the mean values and standard deviations (n=7) of (A) Acetylcholinesterase activity (AChe), (B) Butyrylcholinesterase (BChe), (C) Propionylcholinesterase (PrChe). Statistical significance of the results is compared with the control values (* < 0.05 and ** < 0.01).

3.2 Oxidative stress effects

The biomarkers of oxidative stress responses (GST, CAT, GR and LPO) over a short and long-term exposure to BaP displayed different trends

(**Fig. 3**). GST activity (**Fig. 3A**) exhibited a highly significant inhibition (Kruskal Wallis: $p<0.01$) along time in all concentrations and control, especially if compared to day 1 (Mann-Whitney: $p<0.01$). Significant inhibitions (day 3: positive control, 10, 20 and 100 μg/L) and inductions (day 21: 60 and 100 μg/L) occurred against control (Mann-Whitney: $p<0.05$). CAT activity (**Fig. 3B**) increase along time, especially if compared to Day 1 (Kruskal Wallis: $p<0.01$). Besides, CAT exhibits a clear opposite effect from GST and GR. The most significant inductions against the control occurred at day 1 – 60 and 100 μg/L (Mann-Whitney: $p=0.013$ and $p=0.009$, respectively) – and day 10 – positive control, at 20 and 100 μg/L (Mann-Whitney: $p=0.018$, $p=0.006$ and $p=0.002$, respectively).



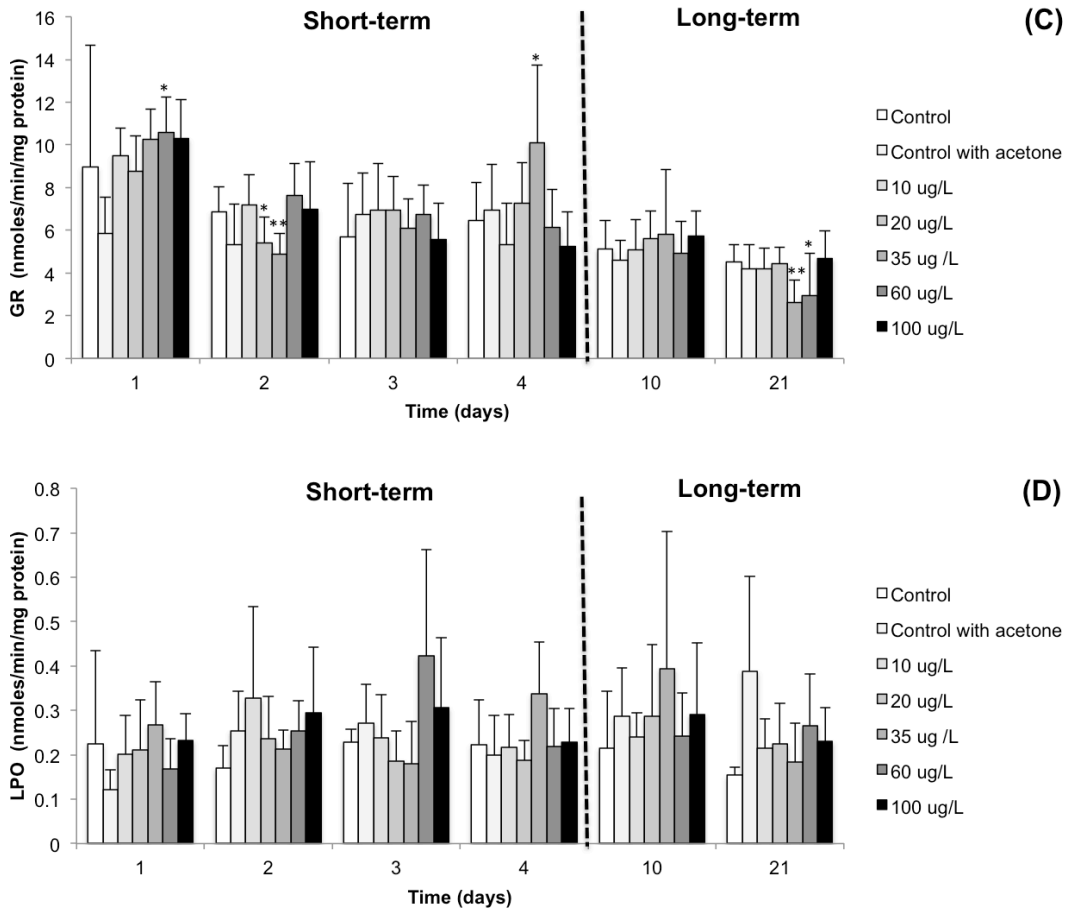


Figure 3. Biomarkers of oxidative stress analyzed in digestive gland of *Mytilus galloprovincialis* in different concentrations of benzo(a)pyrene. Data show the mean values and standard deviations (n=7) of (A) Glutathione S-Tranferase (GST), (B) Catalase (CAT), (C) Glutathione Reductase (GR), (D) Lipid Peroxidation (LPO). Statistical significance of the results is compared with the control values (* < 0.05 and ** < 0.01).

GR activity (**Fig. 3C**) displayed a similar GST pattern, less pronounced but equally significant (Kruskall-Walli: $p<0.01$). BaP-exposed mussels significant inhibitions (day 2: 20 and 35 μg/L; day 21: 35 and 60μg/L) and inductions (day 1: 60 μg/L) occurred against control (Mann-Whitney: $p<0.05$). LPO responses (**Fig. 3D**) exhibited high variance, lacking noticeable trends and relevant statistical results.

3.2 Biomarkers relation with physico-chemical parameters

Pearson correlation (biomarkers response, condition index and physical-chemical parameters) show high impact of abiotic factors in the response of selected endpoints (**Table 1**). The least influenced biomarkers

were BChe and the condition index (CI). Both exposure periods (short and long-term) had different correlations: abiotic factors lacked effects on the endpoints BChe, CI and AChe in the short-term exposure, only in the long-term. Globally, the physicochemical parameters only affected the oxidative stress-related enzymes – excepting the neurotoxicity enzyme PrChe – in both parts of bioassay (**Table 1 and 2 in Supplementary Material**).

Table 1. Pearson correlation coefficient between biomarker responses in digestive gland of *Mytilus galloprovincialis* and physical-chemical parameters in an experimental exposure with benzo(a)pyrene.

	AChe	BChe	PrChe	GST	CAT	GR	LPO	CI
Ammonia	0.090	-0.221	**0.358***	**-0.537****	**0.745****	-0.269	**0.411***	0.008
Nitrite	**0.339***	-0.048	**0.386***	**-0.667****	**0.680****	**-0.556****	**0.396***	-0.078
Temperature	**-0.415***	-0.210	**-0.580****	**0.954****	**-0.561****	**0.840****	**-0.458****	0.261
Conductivity	**0.596****	0.081	**0.435****	-0.727	**0.557****	**-0.771****	**0.362***	**-0.399***
Salinity	**0.582****	0.056	**0.411***	**-0.693****	**0.545****	**-0.739****	**0.354***	**-0.371***
pH	-0.302	-0.310	**-0.478****	**0.841****	**-0.403***	**0.668****	**-0.458****	0.214
OD (mg/L)	**0.441****	**0.434****	0.129	**-0.427***	0.035	**-0.582****	0.008	**-0.601****

Values and asterisks in bold indicate significant relationships (*p<0.05, **p<0.01).

3.2 Relationship between endpoints

The PCA (**Fig. 4**) indicate that the set of variables (biomarkers and C.I.) could be explained by 2 principal components that accounted for 68.51% of total variance (**Table 2**). While all endpoints influence the first component: GST, GR and C.I. weight negatively the component and with 54.78% of the original variance; the second principal component explained 13.73% of the variance: positive values were associated to PrChe, C.I. and LPO and the negative value only to BChe.

Table 2. PCA: Component loadings of the variables for the two principal components in experiment with *Mytilus galloprovincialis* and benzo(a)pyrene.

Variables	***Component 1***	***Component 2***
Eigen values	*4.382*	*1.098*
% of variance	*54.78*	*13.73*
GST	*-0.832*	-
CAT	*0.832*	-
PrChe	*0.787*	*0.353*
AChe	*0.775*	-
GR	*-0.769*	-
CI	*-0.682*	*0.556*
LPO	*0.611*	*0.590*
BChe	*0.604*	*-0.544*

The plot of scores of BaP concentrations and different exposures time for the two principal components (**Fig. 5**) during bioassay displayed different trends. The response of organisms tends to be time-dependent, being more evident for day 1 and day 2 of the experiment: day 1 is influenced by the response of GST and GR (**Fig. 4**); day 3 has a wide variety of response and formed no specific group. The other days lack specific trends, but through time the responses tend to be more differentiate; day 21 form a distinct group in the middle of the clutter, being associated to AChe, PrChe, CAT and LPO. Concerning the concentrations, the responses related to control, positive control and 100 μg/L points tend to be contiguous.

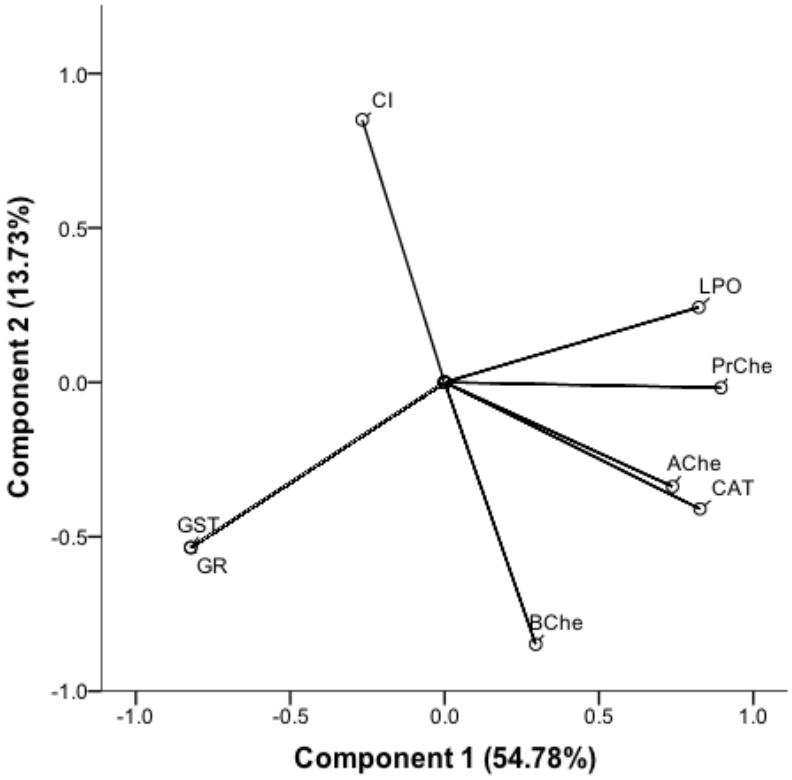


Figure 4. Plot of variable vectors for the two dominant components produced by biomarkers (AChe, BChe, PrChe, GST, CAT, GR, LPO) and C.I. of an exposure with benzo(a)pyrene.

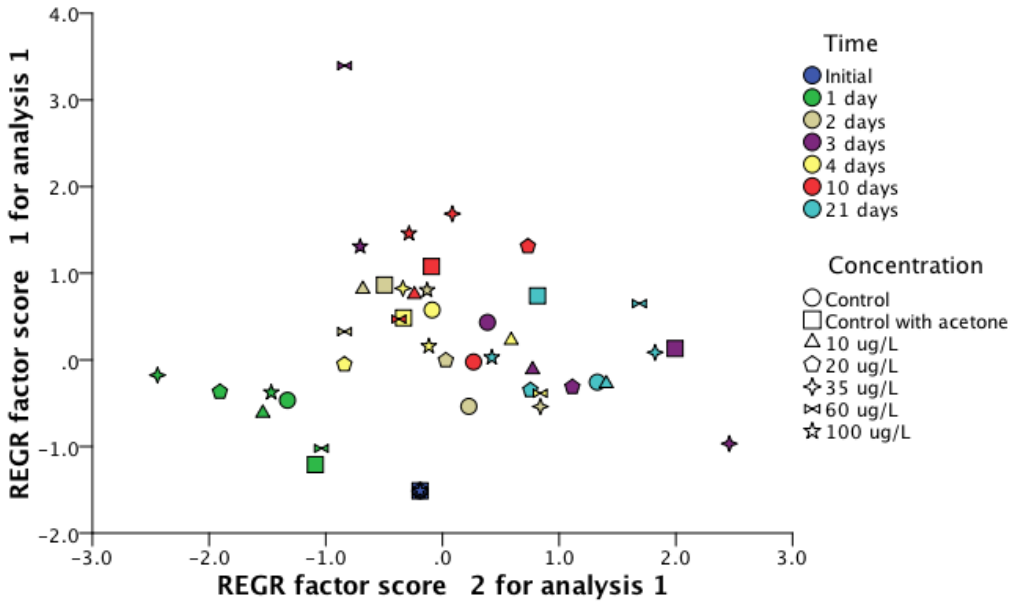


Figure 5. The distribution diagram of the different groups of benzo(a)pyrene concentrations during different experimental periods as a function of the two principal component axis. Principal component loading and total variance associated with each axis are provided in Table 2.

4. Discussion

We found that BaP-exposed mussels showed wide range of responses linked to the physiological pathway of detoxification of BaP and metabolites in the digestive gland. ChEs demonstrate a higher sensibility to BaP through expression of PrChe. Oxidative stress enzymes have different responses over time but no significant effect was detected in LPO levels.

Several studies (Pan et al., 2008; Skarphéinsdóttir et al., 2003; Wang et al., 2011; Xiu et al., 2014) described BaP bioaccumulation in bivalves – fast uptake – and dose-tissue accumulation relation.

Concerning neurotoxicity, we observed that mussels demonstrated a substrate preference towards ASCh, followed by PrSCh and BSCh, which are supported by other studies (Brown et al., 2004; Lau and Wong, 2003; Talesa et al., 2001; Yaqin, 2010). AChe activity showed an inhibition trend in the short-term exposure; Banni et al. (2010) establish a similar pattern after 2 and 3 days of experiment in a similar concentration of BaP, although in our research there is an inhibition of PrChe instead of AChe at 3 days of exposure. Perhaps, this alternation in ChEs inhibition pattern is a mechanism

to prevent the overload of the detoxification pathway and also, damages in mussel's physiology and health status; moreover, pseudocholinesterases (e.g. BChe or PrChe) already were Ache protection-linked, scavenging anti-cholinergic compounds (Masson and Lockridge, 2010; Salles et al., 2006).

AChe main role is neurotransmission regulation, what could be highly sensitive to biotic and abiotic factors (Dimitriadis et al., 2012). In the short-term exposure the abiotic factors lack influence in the AChe responses: no correlation to physico-chemical parameters (**Table 4, Supplementary Material**). However, PrChe was correlated to several abiotic factors: positively (conductivity, salinity and levels of nitrite) and negatively (temperature and pH), what could explain inhibition patterns demonstrate.

The regulation of neurotransmission in bivalves is also associated to valve closure, consequently to food intake and alteration of body size in invertebrates (Duquesne, 2006). Since after 2 days of exposure the CI decayed, especially related to weight of mussels; maybe the inhibitory BaP effect on ChEs will be translated to valves closure and thus, less food intake along time. Unlike Nilin et al. (2012) - linked only PrChe activity to valve closure and metabolism processes in *Cerastoderma edule* – we correlated CI to all three ChEs (**Table 5, Supplementary Material**).

Along BaP-exposure, PrChe – unlike BChe – underwent a xenobiotic-related inhibition in both exposure parts: at long-term exposure the pattern might be direct BaP toxicity or an influence of physico-chemical parameters (**Table 5, Supplementary Material**). While Kekwick (1960) related PrChe – largely unstudied in bivalves – to inflammatory processes; Koelle and Friedenwald (1949) suggested it as AChe substitute in stressors-impairment or inhibition situations: unappropriated for the long-term exposure case.

The influence of ChEs in the enzymatic response of *M. galloprovincialis* is better understood when taking into account separated (short and long-term) analysis of principal components (**Figures 6 to 9, Supplementary Material**).

All three neurotoxicity endpoints are relevant in analysis of principal components of the short-term exposure, independently of the BaP concentration, mainly at day 2 and 4. In the long-term exposure, ChEs lack a higher influence in global response of organisms: while BChe is mostly linked

to post- day 10 exposure, AChe and PrChe are linked to the end of the exposure (day 21).

Furthermore, a clear link arise from the neurotoxicity enzymes and oxidative stress endpoints along exposure (short and long); Melo et al. (2003) speculated that enhanced ChEs activity is concomitant with the presence of high levels of ROS and RNS (nitrogen ones).

Concerning oxidative stress-related endpoints, there was a decrease in activity of GST and GR, and an increase in CAT; no trends were found to LPO. These findings are consistent with previous authors (Akcha et al., 2000; Liu et al., 2014; Livingstone et al., 1990; Maria and Bebianno, 2011; Pan et al., 2009; Wang et al., 2011) reporting BaP induction of the antioxidant defense system: enhancing the ROS formation and/or depleting antioxidant efficiency (Benedetti et al., 2015).

GST showed a biphasic response: activity inhibition followed by induction after 21 days of exposure. Pan et al. (2005) and Liu et al. (2014) showed low-level PAHs exposure inducing the GST activity and vice versa; since our study was conducted with high concentrations the pattern observed could be corroborated with studies mentioned previously. van Ommen et al. (1991) linked the GST inhibition to a competition between endogenous and PAHs biotransformation-related substrates; Pan et al. (2009, 2005) also suggested that high contaminant concentrations might damage the detoxification system or an adaptive response might shift the detoxification pathway promoting another enzyme. GST induction at the end of experiment might be concomitant to a faster excretion of Phase I-related metabolites: future research efforts should focus on the study of the recovery of the detoxification system along time.

Similarly to GST, GR activity decreased over time and Maria and Bebianno (2011) reported the trend. GST and GR are linked through GSH recycling: detoxification pathway via GST might impaired because the declined GSH production by GR; one hypothetical reason for both decreased over time. PCA results for GR and GST relationship show a strong correlation, highly influencing day 1 of exposure. Pan et al. (2009) found that: while GST activity decayed significantly after 12h of exposure; superoxide dismutase

(SOD), CAT, glutathione peroxidase (GPx) – antioxidant enzymes – boosted to eliminate ROS.

Oppositely, CAT activity increase along the exposure, even though the most significant outcomes took place at day 1 and 10. Scott et al. (1991) stated that CAT activity requires NADPH for its maintenance, and also for the proper GR function; thus, a hypothetical competition effect might lead to GR decay. We can verify this opposite relation in PCA results, with a clear pattern (CAT e GR) for both exposures (short and long-term) (**Fig. 6 to 9, Supplementary Material**).

Indifferently, LPO responses lack significance differences over time, implying that oxidative stress enzymes efficiently counteracted the intracellular ROS formation: preventing permanent damages to mussels' physiology. Xiu et al. (2014) found opposite results, where PAHs (BaP, benzo(b)fluoranthene and chrysene) induced LPO levels in *Chlamys farreri* over 21 days of exposure, suggesting that *M. galloprovincialis* cope with a better detoxification system.

During whole exposure time occurred high fluctuations in the enzymatic activities, even in control groups. The excretory system of bivalves produces high ammonia levels and thus, nitrites and nitrates. These metabolites summed with variations of pH, conductivity and other media physico-chemical parameters could shift the availability of contaminants and/or the percentage of unionized ammonia: more toxic to the aquatic organisms (Alabaster and Lloyd, 1982). Oliveira et al. (2014) reported a similar effect for bivalves under starvation, however our organisms were only under starvation for the first 4 days of exposure; Vidal et al. (2002) showed pH inducing alterations the antioxidant enzymes activity in *Corbicula fluminea*, so maybe the slight pH fluctuations in our study caused effects to the enzymatic activities. Regardless, this effect did not exclude the differences observed among control and experimental groups.

Our study suggests that the incorporation of several ChEs in studies related to POP's could be very effective to understand the effect of these contaminants in invertebrates. The oxidative-stress pathway in mussels is significant resilient and was affect by high concentrations of BaP, but no permanent damage was caused.

5.Conclusion

The digestive gland of mussels is the organ responsible for detoxification of xenobiotics in bivalves and apparently high concentrations of BaP has great influence in enzymatic activity of *Mytilus galloprovincialis.* Antioxidant system works to excrete the metabolites originating from Phase I of detoxification and it is easier to conclude that these defenses are properly working conserving the health state of organisms. PCA analysis demonstrates a linkage between oxidative stress and neurotoxicity enzymes, what leads to conclusion that ChEs (especially AChe and PrChe) should be incorporate to monitoring of effects caused by PAHs. Another conclusion is that time of exposure was more important than the concentration used in the response of organisms. To a better understanding of these physiological responses, studies of metabolites and/or genomics should be incorporated in future researches.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

Authors acknowledge Fundação para Ciência e Tecnologia (FCT - Portugal) by the financial support through the grants SFRH/BD/62561/2009 (Jessica C.L. Ladewig), SRH/BD/97877/2013 (Susana Pereira), SFRH/BD/92230/2013 (Marco Fernandes) and the Biology Department of Aveiro University for the tactical support during realization of bioassays and enzymatic analysis.

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SUPPLEMENTARY MATERIAL

Table 3. Pearson correlation coefficients between biomarker responses in digestive gland of *Mytilus galloprovincialis* and physico-chemical parameters in an short-term experimental exposure with benzo(a)pyrene.

	AChe	BChe	PrChe	GST	CAT	GR	LPO	CI
Ammonia	0.114	0.121	0.333	**-0.675****	**0.850****	**-0.481***	**0.443***	-0.104
Nitrite	0.080	0.156	**0.451***	**-0.768****	**0.805****	**-0.646****	0.394	0.033
Temperature	-0.160	-0.131	**-0.704***	**0.971****	**-0.739****	**0.836****	**-0.567****	-0.003
Conductivity	0.394	0.018	**0.686****	**-0.837****	**0.618****	**-0.705****	0.323	0.004
Salinity	0.412	-0.016	**0.670****	**-0.802****	**0.584****	**-0.666****	0.297	0.037
pH	-0.180	-0.285	**-0.679****	**0.866****	**-0.662****	**0.708****	**-0.728***	0.027
OD (mg/L)	-0.039	-0.202	0.323	**-0.660****	0.374	**-0.609****	0.056	-0.153

Values and asterisks in bold indicate significant relationships (*p<0.05, **p<0.01).

Table 4. Pearson correlation coefficients between biomarker responses in digestive gland of *Mytilus galloprovincialis* and physico-chemical parameters in a long-term experimental exposure with benzo(a)pyrene

	AChe	BChe	PrChe	GST	CAT	GR	LPO	CI
Ammonia	0.347	-0.351	**0.619****	**-0.595****	**0.832****	**-0.436***	**0.569****	-0.059
Nitrite	**0.521***	-0.071	**0.593****	**-0.769****	**0.698****	**-0.654****	**0.544***	-0.148
Temperature	**-0.802****	-0.320	**-0.688****	**0.968****	**-0.645****	**0.973****	**-0.574***	**0.618****
Conductivity	**0.617****	0.151	**0.639****	**-0.940****	**0.636****	**-0.898****	**0.554***	**-0.492***
Salinity	**0.585****	0.125	**0.623****	**-0.926****	**0.633****	**-0.876****	**0.551****	**-0.451***
pH	**-0.709****	-0.367	**-0.535***	**0.893****	**-0.504***	**0.878****	**-0.443***	**0.596****
OD (mg/L)	**0.554****	**0.623****	0.271	**-0.528***	0.050	**-0.634****	0.114	**-0.796****

Values and asterisks in bold indicate significant relationships (*p<0.05, **p<0.01).

Table 5. Pearson correlation coefficients between biomarker responses in digestive gland of *Mytilus galloprovincialis* in a experimental exposure with benzo(a)pyrene

	AChe	BChe	PrChe	GST	CAT	GR	LPO
AChe	X						
BChe	**0.506***	X					
PrChe	**0.754****	**0.355***	X				
GST	**-0.445****	**-0.319***	**0.566****	X			
CAT	**0.500****	**0.420****	**0.564****	**-0.684****	X		
GR	**-0.508****	**-0.335***	**-0.487****	**0.834****	**-0.513****	X	
LPO	**0.388****	0.186	**0.579****	**-0.487****	**0.554****	-0.274	X
CI	**-0.458****	**-0.569****	**-0,292***	**0.485****	**-0.583****	**0.494****	-0.186

Values and asterisks in bold indicate significant relationships (*p<0.05, **p<0.01).

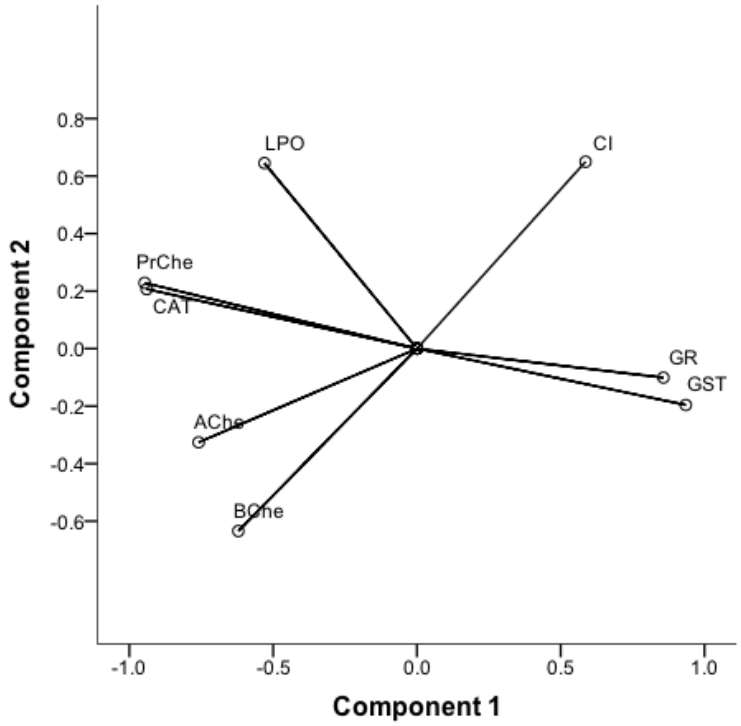

Figure 6. Plot of variable vectors for the two dominant components produced by biomarkers (AChe, BChe, PrChe, GST, CAT, GR, LPO) and C.I. of an short-term exposure with benzo(a)pyrene.

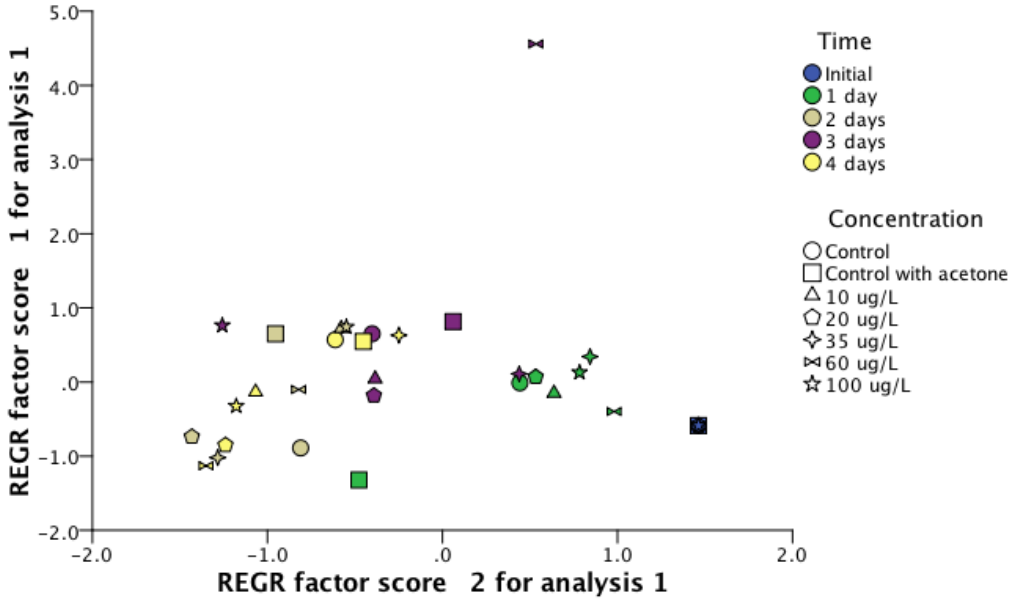

Figure 7. The distribution diagram of the different groups of benzo(a)pyrene concentrations during different experimental periods as a function of the two principal component axis. Principal component loading and total variance associated with each axis are provided in Table 6.

Table 6. PCA: Component loadings of the variables for the three principal components in short-term experiment with *Mytilus galloprovincialis* and benzo(a)pyrene.

Variables	*Component 1*	*Component 2*	*Component 3*
Eigen values	*4.340*	*1.296*	*1.008*
% of variance	*54.249*	*16.195*	*12.602*
PrChe	*0.863*	*0.369*	*-*
CAT	*0.825*	*-*	*-*
GST	*-0.824*	*-*	*0.495*
AChe	*0.731*	*-*	*0.525*
GR	*-0.709*	*-*	*0.572*
LPO	*0.654*	*0.537*	*-*
BChe	*0.647*	*-0.569*	*-*
CI	*-0.595*	*0.674*	*-*

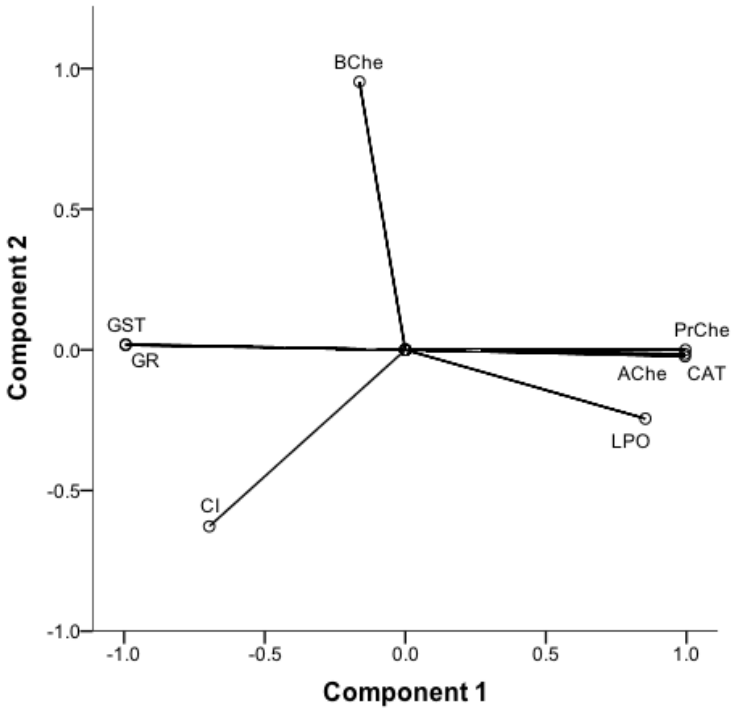

Figure 8. Plot of variable vectors for the two dominant components produced by biomarkers (AChe, BChe, PrChe, GST, CAT, GR, LPO) and C.I. of a long-term exposure with benzo(a)pyrene.

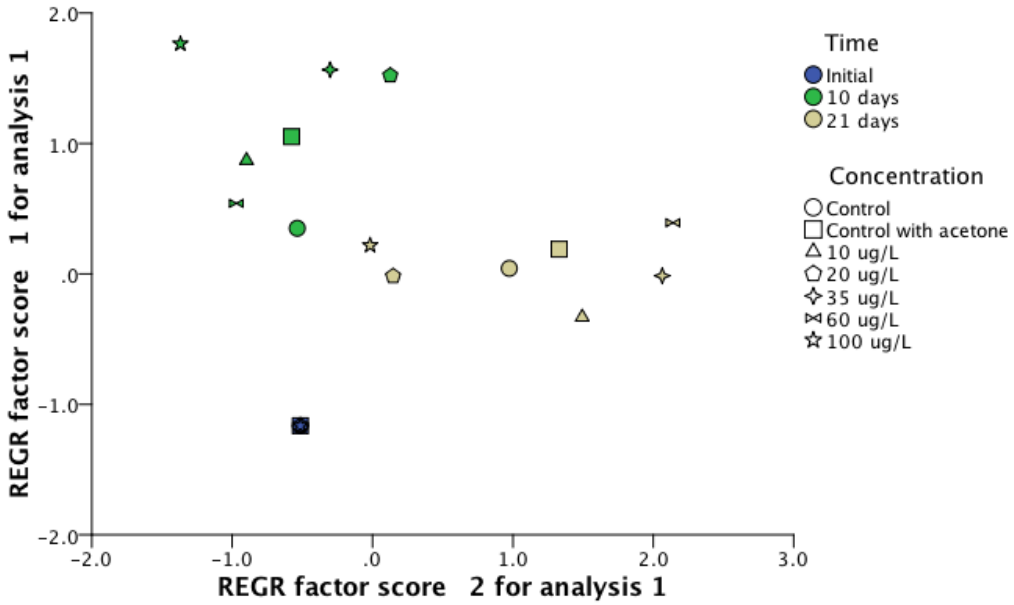

Figure 9. The distribution diagram of the different groups of benzo(a)pyrene concentrations during different experimental periods as a function of the two principal component axis. Principal component loading and total variance associated with each axis are provided in Table 7.

Table 7. PCA: Component loadings of the variables for the two principal components in a long-term experiment with *Mytilus galloprovincialis* and benzo(a)pyrene.

Variables	***Component 1***	***Component 2***
Eigen values	*5.057*	*1.149*
% of variance	*63.210*	*14.366*
GST	*-0.913*	-
GR	*-0.911*	-
AChe	*0.822*	-
PrChe	*0.863*	-
CAT	*0.818*	*0.458*
CI	*-0.716*	*0.391*
LPO	*0.679*	*0.331*
BChe	*0.477*	*-0.759*

CHAPTER

5

EFFECTS OF ENDOSULFAN ON NEUROTOXIC AND ANTIOXIDANT ENZYMES IN MUSSELS (*MYTILUS GALLOPROVINCIALIS*)

Effects of Endosulfan on neurotoxic and antioxidant enzymes in mussels (*Mytilus galloprovincialis*)

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This chapter was submitted as an original article to:

Ecotoxicology and Environmental Safety

Abstract

Mussels are used to biomonitor the effects of several contaminants. The interest in the effects caused by Endosulfan (EDS) is growing in the scientific community, due to its high resilience in aquatic environments. Five concentrations of EDS (0.01, 0.025, 0.05, 0.1 and 0.25 μg/L) plus a control were chosen to assess the response of individuals of *Mytilus galloprovincialis* over 21 days of experiment. The selected endpoints were related to neurotoxicity (AChe, BChe and PrChe), oxidative stress (GST, CAT, GR and LPO) and condition index. Neurotoxicity enzymes demonstrate no inhibitory response; AChe was insensitive to the tested concentrations while BChe showed induction in the short-term exposure and PrChe in the long-term. This pattern suggests that neurotoxicity caused by EDS follows another detoxification pathway. Enzymatic activity of GST and GR declined over time while CAT increased, this trend was associated with the antioxidant system and detoxification pathway of EDS and metabolites. LPO lacked significant effects, implying that oxidative stress-defences of *M. galloprovincialis* are working, and these organisms could deal with environmentally relevant concentrations of EDS. The reaction of the organism as a whole was measured by PCA analysis and initially time exposure was more relevant than the concentrations used, but after 21 days of exposure, the concentrations influenced more the physiological response of mussels. To a better understanding of these responses, slightly higher concentrations should be tested to understand the detoxifications pathways.

Keywords: *Mytilus galloprovincialis*, bivalve, Endosulfan, cholinesterases, neurotoxicity, oxidative stress.

1. Introduction

The use of pesticides has grown exponentially in the past decades and some compounds can be found in aquatic environments and bioaccumulate in organisms along trophic chain (Mahmoud and Loutfy, 2012). Pesticides can enter the aquatic ecosystems through a direct application (spray drift), surface runoff from the soil and through urban and industrial discharge (Deger et al., 2003).

Endosulfan (EDS), a cyclodiene organochlorine pesticide, has been worldwide used for around 50 years, being effective against a broad number of insects and mites (Douthwaite, 1982; Maier-Bode, 1968; OSPAR, 2002; Roberts et al., 2003). Recently (2012), this pesticide use was restricted (UNEP-POPS-COP5, 2011). However, it is still present in remote locations (i.e. the arctic) and, therefore, it has the propensity to undergo long-range transport (Weber et al., 2010). Most environmental protection agencies classified EDS as highly toxic to several aquatic organisms (Sutherland et al., 2004). Some long-term toxic effects of EDS were linked to the activity of sulfate – major metabolite of endosulfan (Day, 1991) – that can persist in natural waters for months (Wan et al., 1995).

Marine bivalves, as *Mitylus galloprovincialis*, are often used as sentinel organisms for various reasons: wide geographical distribution, sessile state filter feeding, and accumulation of water-available xenobiotics. Enzymatic biomarkers are broadly used and recommended to assess the effects of chemical contamination on the physiology of bivalves (Cajaraville et al., 1996; Galloway et al., 2002; Renault, 2011). Even though, little information about biomarkers responses to EDS is available in bivalves.

Cholinesterases (ChEs) are specialized enzymes that play a central role in neurotransmission (Kaufer et al., 1998; Soreq and Seidman, 2001; Vale et al., 2003). The inhibition of acetylcholinesterase (AChe) was previously applied as a specific biomarker in response to organophosphate and carbamate pesticides (Führer et al., 2012; Fulton and Key, 2001; Gagné et al., 2010; Viarengo et al., 2007). Organochloride pesticides (e.g., EDS) are developed to attack the nervous system of target species. Nevertheless, several aquatic organisms (non-target species) exhibit neurotoxicity damages due to the presence of this xenobiotic (Ballesteros et al., 2009a; Da Cuña et

al., 2011; Dutta and Arends, 2003; Pereira et al., 2012; Tao et al., 2013a; Trekels et al., 2012).

The elimination of pollutants in aquatic organisms occurs through two metabolic processes: 1) biotransformation by Phase I (7-ethoxyresorufin O-deethylase – EROD, and cytochrome P450) and Phase II (family of glutathione S-Transferase - GST) enzymes; 2) detoxification-related antioxidant enzymes acting after biotransformation of pollutant. Several authors employed GST to assess the toxicity of EDS in fishes (Ballesteros et al., 2009b; Dorval and Hontela, 2003; Pandey et al., 2001; Salvo et al., 2012) and fewer in bivalves (Tao et al., 2013b). GST is responsible to the conjugation of electrophilic compounds as a sub-product of Phase I of detoxification; the toxicity of several compounds can be modulated by its induction (Van der Oost et al., 2003). Antioxidant enzymes like catalase (CAT) and glutathione reductase (GR) attack and eliminate reactive oxygen species (ROS), which are generated during the contaminants detoxification-related process and could be more dangerous to organisms than the pollutant itself (Livingstone et al., 1992). Failure of antioxidant defenses can lead to oxidative damage, including enzyme inactivation, protein degradation, DNA damage and, lipid peroxidation (LPO) (Halliwell and Gutteridge, 1999; Tao et al., 2013b).

In this study, we aimed to assess the sublethal effect of endosulfan on the physiological response of *Mytilus galloprovincialis* through several endpoints, in a short and long-term exposure. We hoped to better understand the detoxification pathway of ecologically relevant concentrations of endosulfan and contribute to validate the use of neurotoxicity and oxidative stress enzymes in the context of organochlorine pesticides.

2. Materials and Methods

2.1 Chemicals and preparation of solutions

Endosulfan (EDS) (99.4% purity) was purchased from Sigma Aldrich (Germany). Endosulfan stock solution (10 mg/L) was set, kept in the fridge and protected from the light. Dilutions for the test solutions were performed with artificial saltwater immediately prior to test's set up.

2.2 Organism selection and Experimental design

M. galloprovincialis specimens (n=300) were collected from Aveiro Estuary (July of 2013) and scrubbed from fouling organisms. Then, mussels were acclimated (7 days) (19ºC, water changed each 3 or 4 days), kept under natural light and starvation in the short-term exposure. Organisms selected for EDS experiment were exposed to control and 5 concentrations (0.01, 0.025, 0.05, 0.1, 0.25 µg/L). Test conditions were similar to the acclimation ones; however in the long-term exposure water was entirely renewed each four days and mussels feed with homogenized fish food (TetraMin, TetraWerke, Melle, Germany) each other day. A set (n=10) of digestive gland tissue was dissected and regarded as time sampling = 0 days (initial). In the next sampling periods (1, 2, 3, 4, 10 and 21 days) 7 individual fractions per treatment were dissected and snap-frozen (-80ºC) in microtubes until enzymatic activity analysis.

2.2.1 Body Condition Index

The shells of each organism were measured (i.e. length, width and heights) to calculate condition indices of the individual bivalve, based upon the following equations:

$$\text{Internal volume} = 3/4 * \text{length} * \text{width} * \text{height}$$

$$\text{Condition Index (CI)} = \text{Dry weight (g)} / \text{Internal Volume (cm}^3\text{)}$$

Dry weight was obtained through the dissection of organisms: the whole soft tissue pool of 10 individuals was dehydrated (45ºC, 48h) and weighted.

2.3 Physico-chemical variables

Several physical-chemical parameters (e.g. temperature, conductivity, dissolved oxygen, pH and salinity) were monitored through a multi-parameter sensor along the experiment. Nitrite and ammonia were analyzed

(UNEP/IOC/IAEA, 1991) to confirm the tolerance levels of the bivalves (Epifanio and Srna, 1975) and the lack bias in the effects of EDS.

2.4 Biomarkers analysis

Digestive gland of mussels was homogenized in phosphate buffer (0.01 M, pH 7.4) and centrifuged (10000 g, 20 min.) to isolate the post-mitochondrial supernatant (PMS) (Howcroft et al., 2011). Enzymatic biomarkers were selected to determine neurotoxicity and oxidative stress endpoints and their activities were spectrophotometrically measured (Thermo Scientific Multiskan® Spectrum) in 96-well microplates.

2.4.1 Neurotoxicity enzymes

Three esterases enzymes were selected: acetylcholinesterase (AChe), butyrylcholinestrease (BChe) and propionylcholinesterase (PrChe). ChEs activity (λ = 414 nm) was quantified – 50 µL of PMS and 250 µL of reaction solution (0.075 M choline substrate and 10 mM 5,5-dithiobis-2-nitrobenzoic acid (DTNB) in K-Phosphate buffer (0.1 M, pH 7.2)) – through supervision of absorbance's increase (each 20 sec in 3 min). Substrate analogues – acetylthiocholine iodide (ASCh), butyrylthiocholine iodide (BSCh) and propionylthiocholine (PrSCh) – were degraded as described by Ellman et al. (1961) and modified by Guilhermino et al. (1996).

2.4.2 Oxidative Stress enzyme

Four endpoints were chosen to assess the oxidative stress produced through exposure of EDS. Three redox cycle-related enzymes (CAT, GR, GST) and one oxidative stress outcomes-related enzyme (LPO) were assessed. CAT activity (λ = 240 nm) was quantified – 150 µL of PMS and 150 µL of reaction solution (30 mM hydrogen peroxide (H_2O_2) in K-phosphate buffer (K-phosphate) (0.05 M, pH 6.5)) – monitoring the absorbance's decrease (each 10s in 5.30 min), i.e. H_2O_2 decomposition (Clairborne, 1985). GR activity (λ = 340 nm) was determined – 100 µL of PMS and 200 µL of reaction solution (0.1 mM glutathione oxidase (GSSG), 0.2 mM nicotinamide adenine dinucleotide phosphate (NADPH) and 0.5 M of diethylene triamine pentaacetic acid (DTPA) in K-phosphate buffer (0.05 M, pH 7.0)) – monitoring

the absorbance's decrease (each 20 sec in 5 min), i.e. GSSG degradation (Cribb et al., 1989). GST activity (λ = 340 nm) was measured – 100 µL of PMS and 200 µl of reaction mixture (20 mM reduced glutathione (GSH)) and 120 mM 1-chloro- 2.4-dinitrobenzene (CDNB) in K-phosphate buffer (0.1 M, pH 6.5) – checking the absorbance's increase (each 20s in 5 min), as described by Habig and Jakoby (1981) and modified by Frasco and Guilhermino (2002). LPO levels were measured (λ = 532 nm) through the generation of Thiobarbituric acid (TBARS)-malondialdehyde (MDA) reactive species. PMS (150 µL) and reaction mixture (12% of trichloroacetic acid (TCA) in ultrapure water, 60mM Tris–HCl with 0,1 mM DTPA and 0.73% 2-thiobarbituric acid (TBA) in ultrapure water) were mixed, heated (1h, 100ºC) and particulate materials removed by centrifugation (Ohkawa et al., 1979). Enzymatic activities were determined in triplicate, expressed as nanomoles of substrate hydrolyzed per minute per mg of protein (nmol min^{-1} mg protein). Protein concentration (λ = 595 nm) was quantified in quadruplicate according to Bradford (1976), using albumin as standard.

2.5 Statistical analysis

All data were statistically analyzed with SPSS 21.0 software. All data failed normality (Kolmogorov-Smirnov and Shapiro-Wilk tests) and/or homogeneity (Levene's test). Therefore, different experimental exposures and periods were compared with Kruskal-Wallis and Mann-Whitney U as a post-hoc test. Relationships between biomarkers and physical-chemical parameters were evaluated through Pearson correlations. Principal Component Analysis (PCA) with orthogonal rotation (varimax) was executed with two types of variables: biomarkers (AChe, BChe, PrChe, GST, CAT, GR and LPO) and condition index values (CI). Only eigenvalues over 1 and component values over 0.30 were considered; all variables were standardized by Z-score method (mean=0 and standard deviation=1).

3.Results

3.1 Body Condition Index

Condition Index for *M. galloprovincialis* (**Fig. 1)** exposed to EDS demonstrates a slight decrease over time. Significant values against control occurred in the long-term exposure: 0.025 μg/L at 10 days (Mann-Whitney: p=0.023) and 0.25 μg/L at 21 days (Mann-Whitney: p=0.019).

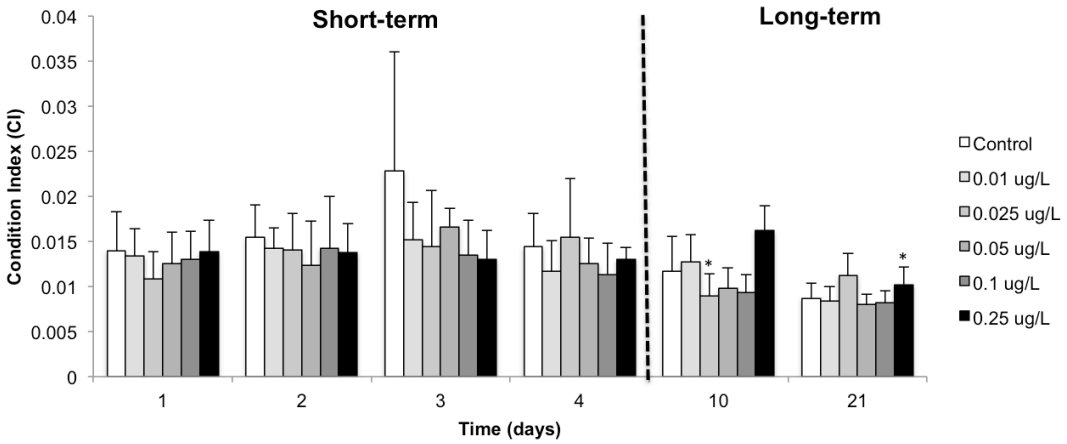


Figure 1. Condition Index (C.I.) of *Mytilus galloprovincialis* in different concentrations of endosulfan. Data show the mean values and standard deviations (n=7). Statistical significance of the results is compared with the control values (* < 0.05).

3.1 Neurotoxicity effects

The neurotoxicity response of mussels exposed to EDS (**Fig. 2**) show an induced activity over exposure time. Biomarkers were expressed in distinct levels for each ChE: AChe > PrChe > BChe, consecutively by enzymatic preference. AChe (**Fig. 2A**) was induced at day 1 for 0.025 μg/L (Mann-Whitney: p=0.025). BChe activity (**Fig. 2B**) increased against the control at day 3 for 0.01 μg/L (Mann-Whitney: p=0.028). PrChe (**Fig. 2C**) was affected in the long-term exposure only, at day 21: 0.05 and 0.25 μg/L (Mann-Whitney: p=0.028 and p=0.019 subsequently).

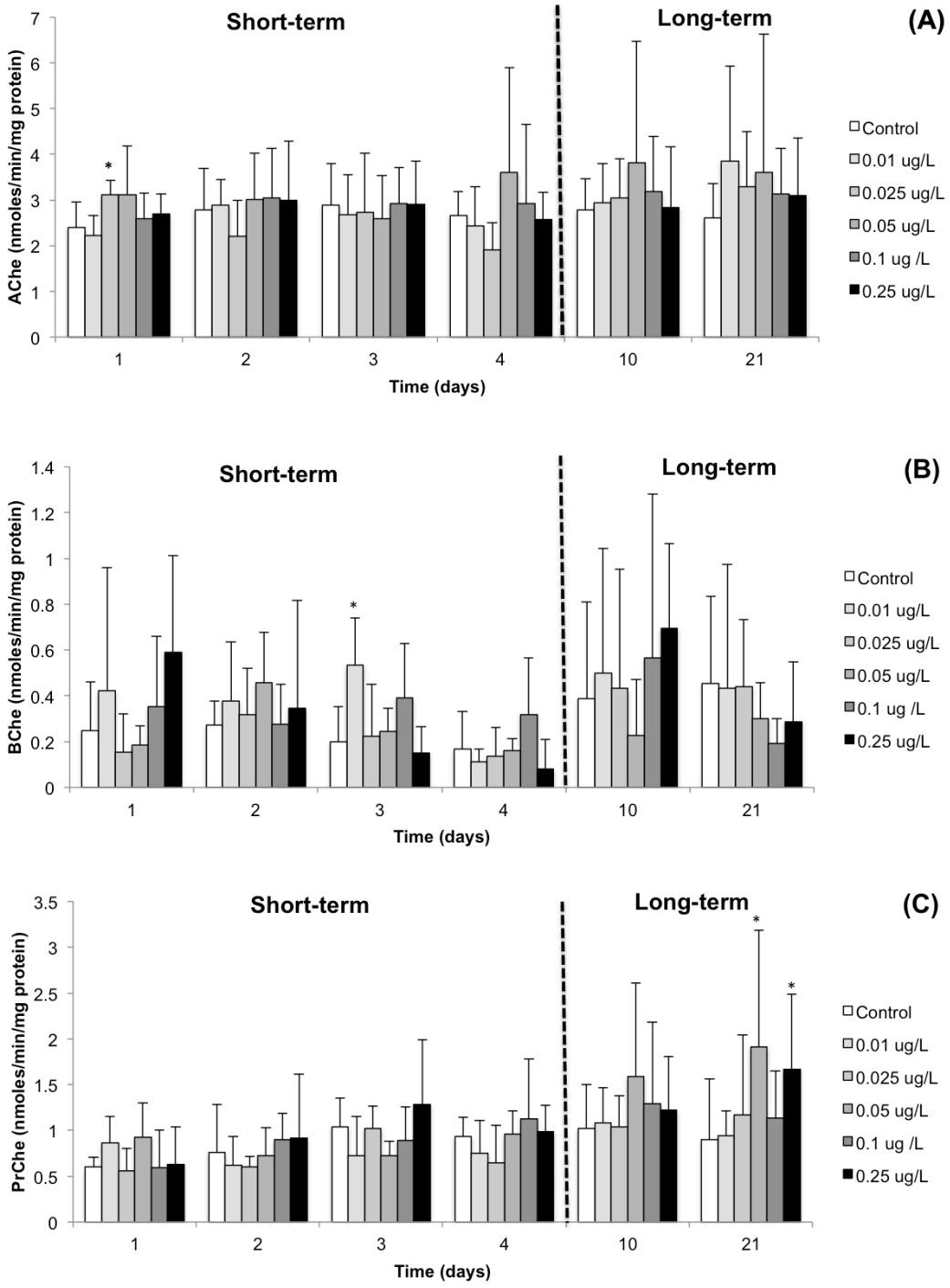
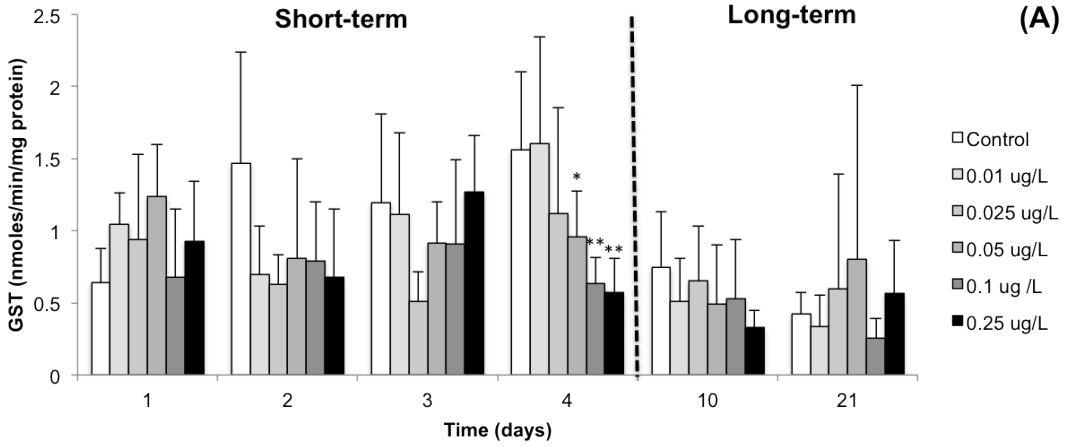


Figure 2. Biomarkers of neurotoxicity analyzed in digestive gland of *Mytilus galloprovincialis* exposed to different concentrations of endosulfan. Data show the mean values and standard deviations (n=7) of (A) Acetylcholinesterase activity (AChe), (B) Butyrylcholinesterase (BChe), (C) Propionylcholinesterase (PrChe). Statistical significance of the results is compared with the control values (* < 0.05).

3.2 Oxidative stress effects

The oxidative stress-related enzymes (GST, CAT, GR, LPO) show different trends over exposure time (**Fig. 3**). GST activity (**Fig. 3A**) exhibited an inhibition on day 4 for the highest concentrations: 0.05, 0.1 and 0.25 μg/L (Mann-Whitney: p=0.018, p=0.002 and p=0.003, successively). In the long-term exposure, GST activity decreased, when compared to short-term exposure, even in the control group (Kruskal Wallis: p<0.05). CAT (**Fig. 3B**) shows the same pattern until day 3 of exposure. At day 4 the activity was inverted, becoming significantly higher (or lower?) after 10 days of exposure at 0.01, 0.05, 0.1 and 0.25 μg/L (Mann-Whitney: p=0.002, p=0.016, p=0.041 and p=0.000 respectively). GR activity (**Fig. 3C**) exhibited the same pattern as GST, with an inhibition at day 4 for 0.05 and 0.25 μg/L (Mann-Whitney: p=0.013 and p=0.003, subsequently) and decay in the activity in the long-term exposure (Kruskal-Wallis: p<0.01). Levels of LPO (**Fig. 3D**) lack significant trends, and the response remained constant, with small variations over the exposure time.



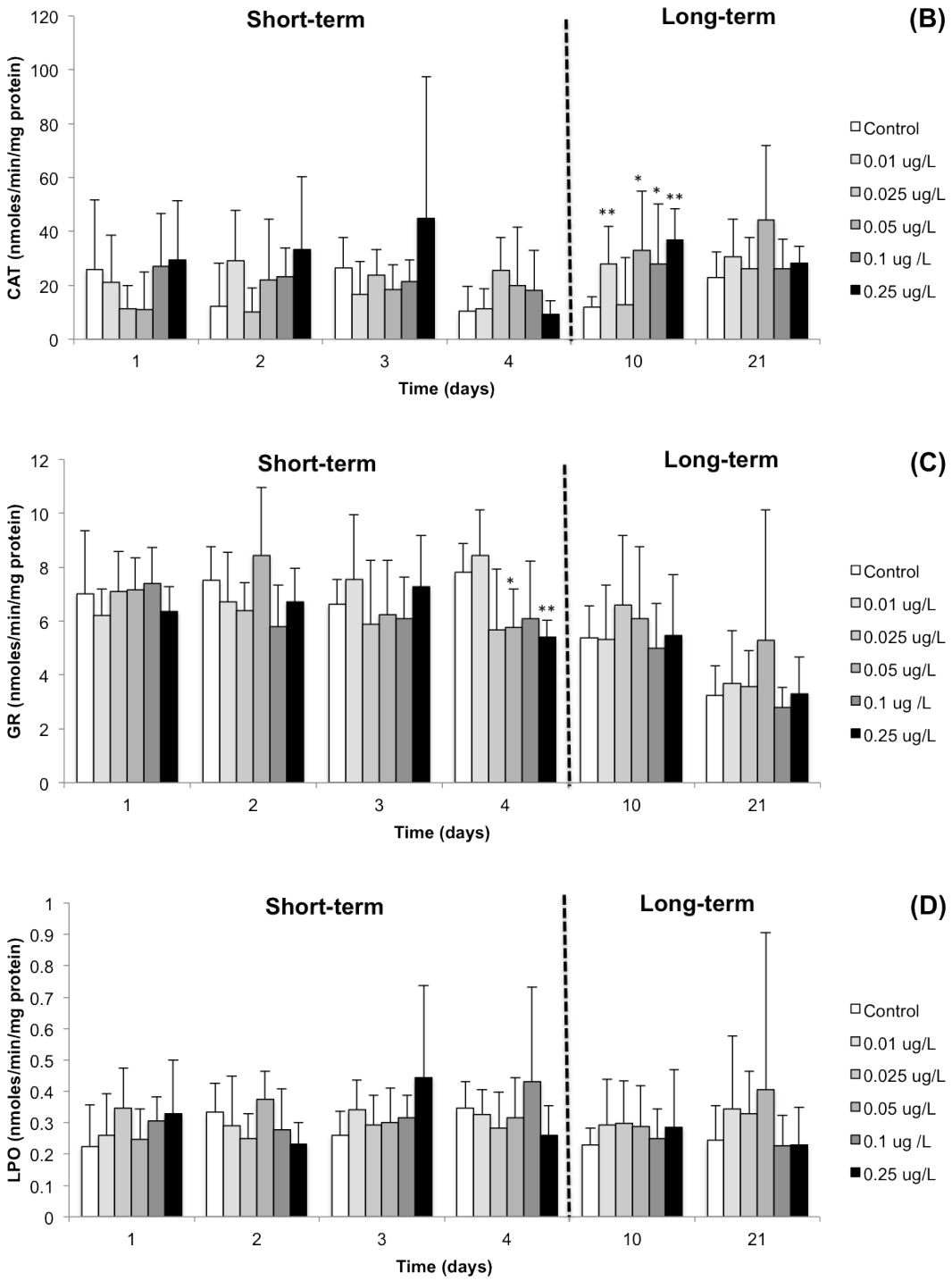


Figure 3. Biomarkers of oxidative stress analyzed in digestive gland of *Mytilus galloprovincialis* exposed to different concentrations of endosulfan. Data show the mean values and standard deviations (n=7) of (A) Glutathione S-Tranferase (GST), (B) Catalase (CAT), (C) Glutathione Reductase (GR), (D) Lipid Peroxidation (LPO). Statistical significance of the results is compared with the control values (* < 0.05 and ** < 0.01).

3.2 Biomarkers relation with physico-chemical parameters

Pearson correlation for the selected endpoints (biomarkers, condition index) and physico-chemical parameters (**Table 1**) show an impact of the abiotic factors in the response of the selected biomarkers, especially for the neurotoxicity enzymes. ChEs are greatly correlated to conductivity, oxygen and salinity (positively for AChe and PrChe, negatively for BChe). The oxidative stress enzymes are less related to these factors, lacking any effects in GST and LPO. The CI response seems to be significantly negative correlated with ammonia, conductivity, pH and dissolved oxygen (DO).

Table 1. Pearson correlation coefficient between biomarker responses in digestive gland of *Mytilus galloprovincialis* and physico-chemical parameters in an experimental exposure with endosulfan.

	AChe	BChe	PrChe	GST	CAT	GR	LPO	CI
Ammonia	**0.384***	-0.111	0.310	-0.154	-0.183	0.031	0.096	**-0.370***
Nitrite	-0.149	**-0.537****	-0.247	**0.546****	**-0.628****	**0.691****	0.214	0.198
Temperature	-0.059	0.104	-0.277	0.286	-0.319	**0.545****	0.126	0.239
Conductivity	**0.414***	**-0.491****	**0.418***	0.059	**-0.435***	0.212	0.208	**-0.429***
Salinity	-0.240	**-0.379***	**-0.434***	0.313	-0.276	0.208	0.214	0.239
pH	**0.571****	**-0.749****	**0.464****	0.136	**-0.362***	0.205	0.456*	**-0.589****
OD (mg/L)	**0.391***	**-0.464****	**0.415****	0.065	**-0.416***	0.217	0.212	**-0.396***

Values and asterisks in bold indicate significant relationships (*p<0.05, **p<0.01).

The correlation among endpoints (biomarkers and CI) (**Table 2**) shows a mix of responses. Antioxidant enzymes are correlated with neurotoxin enzymes, lacking any easily identifiable pattern. As expected for biomarkers belonging the same effect group (neurotoxicity and oxidative stress) and protein family (esterases and phase II enzymes), their response during the exposure period followed the same trend, as AChe and PrChe (positive correlation); GR and GST (positive correlation). Conversely, both AChe and PrChe have a negative correlation with CI.

Table 2. Pearson correlation coefficients between biomarker responses in digestive gland of *Mytilus galloprovincialis* in an experimental exposure with endosulfan.

	AChe	BChe	PrChe	GST	CAT	GR	LPO
AChe	X						
BChe	**-0.310***	X					
PrChe	**0.670****	-0.234	X				
GST	-0.199	**-0.387***	-0.219	X			
CAT	0.135	**0.367***	**0.353***	**-0.356***	X		
GR	-0.120	-0.289	**-0.327***	**0.691****	**-0.334***	X	
LPO	**0.402****	-0.261	0.251	**0.343***	0.072	0.391*	X
CI	**-0.611****	0.221	**-0.509****	**0.337***	0.030	0.287	-0.249

Values and asterisks in bold indicate significant relationships (*p<0.05, **p<0.01).

3.2 Relationship between endpoints

The result of the PCA analysis (**Fig. 4**) suggests three principal components to explain the variability of all data (biomarkers and CI), and it explains 77.67% of the total data variance (**Table 3**). The PC1 explains 34.46% of the original variance and undergoing to positive (PrChe, AChe and CAT) and negative (CI, GST and GR) influences. PC2 accounts for 29.28% of total variance: positive values associated to AChe, GST, GR, LPO and negative values to CI, BChe and CAT. PC3 explained 13.94% of the variance, being only positively impacted by CI, BChe, LPO and CAT.

Table 3. PCA: Component loadings of the variables for the three principal components in an experiment with *M. galloprovincialis* and endosulfan.

Variables	***Component 1***	***Component 2***	***Component 3***
Eigen values	*2.756*	*2.342*	*1.115*
% of variance	*34.45*	*29.28*	*13.94*
PrChe	*0.808*	-	-
AChe	*0.750*	*0.478*	-
CI	*-0.733*	*-0.302*	*0.333*
GST	*-0.627*	*0.614*	-
GR	*-0.618*	*0.603*	-
BChe	-	*-0.753*	*0.364*
LPO	-	*0.710*	*0.502*
CAT	*0.454*	*-0.368*	*0.721*

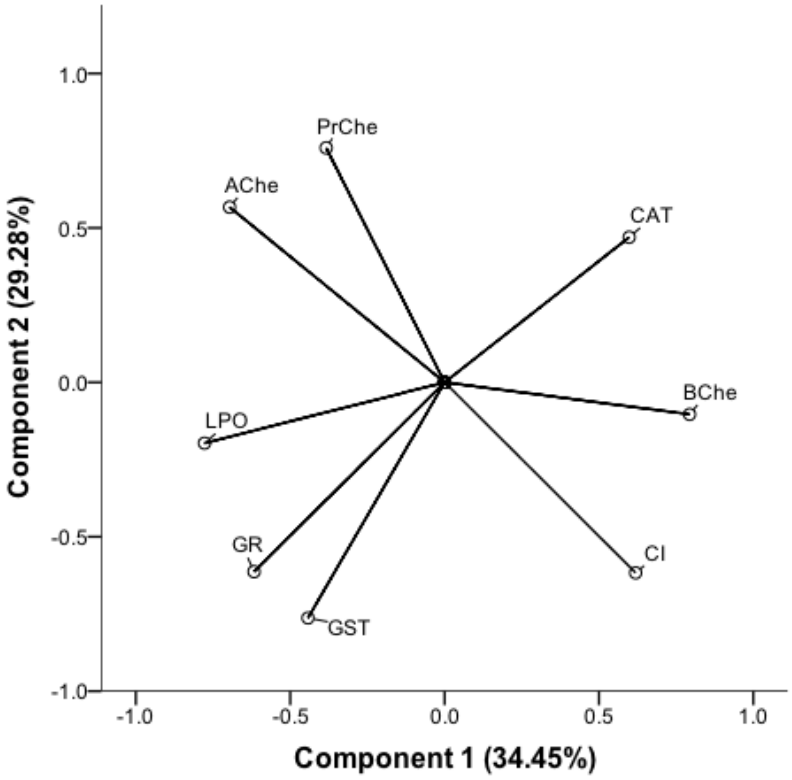


Figure 4. Plot of variable vectors for the two dominant components produced by biomarkers (AChe, BChe, PrChe, GST, CAT, GR, LPO) and C.I. of an exposure with endosulfan.

Plot of scores for PC1 and PC2 from different concentrations of EDS and time exposure (**Fig. 5**) showed distinct trends. The response of organisms implies a time dependency, however controls and the lowest EDS concentrations (0.01 μg/L) grouped together. In the short-term exposure, highest concentrations (0.1 and 0.25 μg/L) tend to cluster closely. Contrary, in the long-term exposure the response also shows a concentration-dependency: after 10 and 21 days of exposure occurs higher difference for each concentration. While the outcome of day 10 and 21 is utterly influenced by AChe and PrChe (**Fig. 4**), in the short-term exposure each day is more associated with a distinct endpoint.

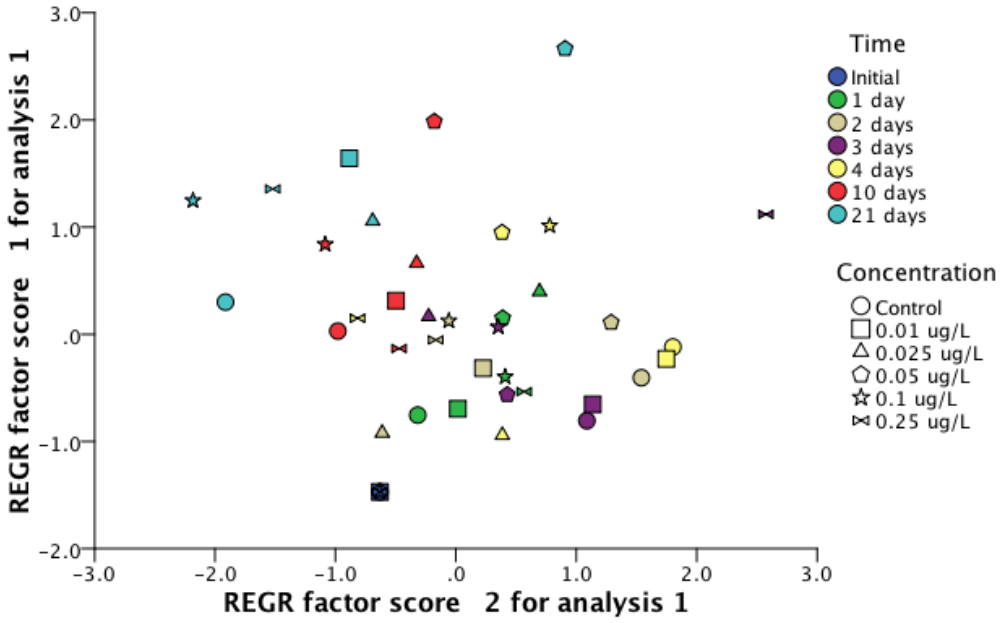


Figure 5. The distribution diagram of the different groups of endosulfan concentrations during different experimental periods as a function of the two principal component axis. Principal component loading and total variance associated with each axis are provided in Table 3.

4. Discussion

We assessed both short and long-term sublethal effects of EDS on the physiological response of *M. galloprovincialis* through a panel of biochemical and individual-level endpoints. The EDS prompted distinct responses across the selected biomarker panel.

Ches were induced at different time exposures: AChe almost lacked any response to the concentrations of EDS, BChe was induced in the short-term exposure and PrChe in the long-term exposure. The oxidative stress induced different responses over-time, but the antioxidant system seemed to be working properly, since LPO levels lacked any damage.

Regarding neurotoxicity results, *M. galloprovincialis* showed a substrate preference for ASCh followed by PrSCh and BSCh, corroborated by Brown et al. (2004), Lau and Wong (2003), Talesa et al. (2001), Yaqin and Hansen (2010). Contrary to other EDS studies (Dutta and Arends, 2003; Tao et al., 2013a; Wu et al., 2011), our lacked any inhibitory trend in all ChEs. Similarly, Ballesteros et al. (2009a) and Da Cuña et al. (2011) reported an

indifferent response of ChE subjected to sublethal concentrations of EDS. Some possible explanations arose for this AChe insensitivity: a different neurotoxicity pathway for this pesticide. For instance an association with the inhibition of neurotransmitters, such as GABA-induced Cl^- flux and $GABA_A$ channels (Vale et al., 2003); or its correlation with the increased PrChe activity over time (Pearson: $p<0.01$). AChe and PrChe are linked in PCA results, both influencing the whole organism response, especially in the long-term exposure (day 10 and 21). All ChEs displayed an induction at some point of exposure to different EDS concentrations. The increasing ChE activity induced by pesticides in laboratory bioassays was also observed in diverse aquatic organisms: grass shrimp larvae, *Palaemonetes pugio* (Key and Fulton, 1993); freshwater mussels, *Elliptio complanata* (Moulton et al., 1996); tadpoles of *Rhinella arenarum* (Rosenbaum et al., 2012); and in the liver of rosy barb, *Puntius conchonius* (Gill et al., 1990). Carboxylesterases (CES) and BChe apparently play a protective role in anticholinesterase intoxication: they remove a significant amount of pesticide and thus, avoiding its arrival in the target AChe site (Sanchez-Hernandez, 2007). For this reason, several authors (Sanchez-Hernandez and Wheelock, 2009; Wheelock et al., 2005) suggest the combined monitoring of CES and ChE activities could provide a more useful indication of pesticide neurotoxicity (Kristoff et al., 2012). Concerning BChe activity decreased compared to other ChE forms, but lacking significance for the range of selected concentrations. BChe could be acting as a molecular decoy of EDS and promoting catalysis of the other ChE forms in the response to the contaminant (Kaufer et al., 1998).

Pertaining antioxidant enzymes trends: GST and GR activity decreased, CAT activity increased, and LPO lacked any trends – suggesting a highly competent antioxidant detoxification system. Ballesteros et al. (2009b) found a similar GST inhibition pattern for *Jenynsia multidentata* exposed to EDS. Although the GST inhibition in response to pesticides is rare, the same trend was reported to diverse aquatic organisms: freshwater snail, *Lymnaea palustris* (Baturo and Lagadic, 1996); embryos of zebrafish, *Danio rerio* (Wiegand et al., 2001); freshwater mussel, *Anodonta cygnea* (Robillard et al., 2003); and Mozambique tilapia, *Oreochromis mossambicus* (Kavitha and Venkateswara Rao, 2009). This result can fit in one of the following

hypotheses: 1) metabolites produced in Phase I of detoxification could be competing with SH substrates for the active sites on the GST enzyme (Egaas et al., 1999); 2) inhibition of the synthesis of GST proteins at molecular levels, lowering enzymatic activity (Gallagher and Sheehy, 2000); 3) different isoforms of GST, as Boutet et al. (2004) demonstrated in bivalves that the exposure length and the type of contaminant can differentiate the expression of some GST's isoforms.

According to Baturo and Lagadic (1996), GST tends to undergo induction when exposed to high concentrations of xenobiotics in laboratory bioassays; oppositely, mesocosm or contaminants at environmental relevant concentrations lack such trends. Comparatively, our study concentrations are environmentally relevant, being lower than the values available in the literature, which could better explain our results. Moore et al. (1987) and Richardson et al. (2008) found that the induction of GST activity related to organochloride pesticides only occurred in depuration period; thus, maybe no oxidative stress response happens to the length of EDS exposure.

Lower production of GSH by GR might elucidate the overall decay in GST activity. GST and GR correlate strongly and co-depend of one another to proper function. GR role is to maintain the intracellular concentration of GSH with consumption of NADPH. GR induction is often associated as a biomarker of oxidative stress (Stegeman et al., 1992; Zhang et al., 2004); controversially, in our study occurred GR inhibition after 4 days of exposure and its activity declined over time. The change in the intracellular NADPH availability might clarify this pattern (Ballesteros et al., 2009b; Moreno et al., 2005; Zhang et al., 2004). GR decrease could lead to GSH depletion and thus, enhance the toxic potential of xenobiotics, producing oxidative species (Babich et al., 1993).

The amplification of antioxidant defenses is expected since pesticides could increase ROS levels, thereby demanding for enzymes to cope with oxidative damage (Trekels et al., 2012; Walker et al., 2004). In the long-term exposure CAT was induced, contrary to other studies (Atif et al., 2005; Ballesteros et al., 2009b; Pandey et al., 2001; Salvo et al., 2012). CAT removes H_2O_2 produced by Superoxide Dismutase (SOD) enzyme, being metabolized to O_2 and water (Van der Oost et al., 2003). The detoxification EDS pathway could produce superoxide anions in excess, increasing the CAT

activity. The oxidative defense system of *M. galloprovincialis* was very efficient against EDS and metabolites-related ROS. The lack of response of LPO levels could corroborate this conviction, however the LPO level's induction in oxidative stress-related conditions occurred in other studies (Atif et al., 2005; Ballesteros et al., 2009b; Dorval and Hontela, 2003; Hincal et al., 1995; Pandey et al., 2001; Tao et al., 2013b). Nonetheless, these studies worked with higher concentrations than ours, so that could explain this induction.

Our study suggests that ecological relevant concentrations of EDS lacked permanent damage to the physiological functions in *M. galloprovincialis*. The antioxidant defense system of mussels seemed very resilient and the neurotoxicity revealed a possibility of a different EDS neurotoxicity pathway. EDS can be associated with the organic matter and remain in the sediments for years (Weber et al., 2010), leading to acute pulses of contamination; thus, higher concentrations should be tested to a better understanding of the detoxification pathway of this organochloride pesticide.

5.Conclusion

Low concentrations of EDS lacked ChEs activity inhibition, leading us to conclude that these enzymes are not suitable to be associated with organochloride pesticides. Antioxidant system excreted efficiently ROS produced by EDS metabolites or by Phase I of detoxification; thus, we conclude that these defenses work properly to conserve the health status of the organisms. PCA analysis demonstrates a higher relevance on the length of exposure, however, after 21 days the concentrations began to be essential to the response of organisms. Higher concentrations and longer exposure periods should be tested and a recovery period should be incorporated in future studies, due that the response of some organisms could take more time to occur and the initial response could lead to misinterpretations.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

Authors acknowledge Fundação para Ciência e Tecnologia (FCT - Portugal) by the financial support through the grants SFRH/BD/62561/2009 (Jessica C.L. Ladewig), SRH/BD/97877/2013 (Susana Pereira), SFRH/BD/92230/2013 (Marco Fernandes) and the Biology Department of the University of Aveiro for the tactical support during realization of bioassays and enzymatic analysis.

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CHAPTER

6

GENERAL DISCUSSION AND FINAL REMARKS

1. General Discussion and Final Remarks

The main objective of this study is to assess the effects of different pollutants (PAHs and pesticides) on physiological parameters of bivalve species from two locations (Mexico and Portugal) of the Northern Hemisphere. This was achieved relying in *in situ* and laboratory experiments with four different species of bivalves (*Mytillus galloprovincialis, Crassostrea virginica, Rangia cuneata* and *Corbicula fluminea*).

1.1 Comparison between Estuarine Ecosystems

Regarding the estuarine experiments, there is a clear difference when comparing endpoints results alone (**Chapters 2 and 3**), especially because they were not performed at the same year, have different degrees of contamination and the physiology and behavior of mussels and oysters differs until certain point. Nonetheless, when comparing the results of the PCA (**Figures 7-8 at Chapter 2; Figures 9-10 at Chapter 3**) it is possible to see some resemblances, after 30 days of experiment the organisms have similar results and the overall response tends to be more time-related, unfortunately there is a discrepancy in the number of endpoints performed in both experiments. When taking into consideration the four endpoints (AChe, GST, CAT and CI) in common (**Tables 1 and 2**; **Figures 1 to 4, Supplementary Material**) it is easy to notice that the "control" points (Point 5 to Términos Lagoon and Point 1 to Aveiro Estuary) tend to cluster together. According to Amiard-Triquet, Amiard, & Rainbow (2012) the method chosen (mesh bags) to perform the experiment did not affect the food availability and consequently the variation in the organism response could be related more to seasonal fluctuation than to the caging process itself.

In Aveiro the response is more time-related than in Términos, this could be a seasonal effect, especially because after 90 days of experiment (mid-summer season) the endpoint that influence the response is the CI. Some studies linked an effect of spring/summer to the physiological response of organisms and advice to take samples from late autumn/early winter, when bivalves are in a more stable physiological state (OSPAR, 2010; Vidal-Liñán and Bellas, 2013). A multibiomarker approach with bivalves in the Ria

Fomosa Lagoon registered stressful condition in the summer months and an effect of abiotic factors in the high biomarker variability (Cravo et al., 2009). In estuarine ecosystems where contaminants occur in mixtures results may not be easily understood and interactions among pollutants as well as with biological systems will influence the effect of other environmental compounds on the organism response (Solé, 2000).

The variety of responses after 30 days of experiment in Términos could be linked to the end of the rainy season, in the last two samplings there is a more universal response, with close to none differences among stations and time. A study of Fang, Fang, Lee, Ko, & Baker (2006) detected a higher level of contaminants from areas under the influence of rivers in the wet season, what could be related to the flush out during the rainfall. This is a possible explanation to our results to Términos Lagoon, where half of the experiment (initial and 48 days sample) was performed in the rainy season and the following sample dates in the dry season.

The differences pointed out between Temperate and Tropical ecosystems could be related to the reproductive period. There is a more orderly arrangement in the data from Aveiro, what could be related to the spawning season, since the experiment took place from early spring to mid summer. According to Fearman & Moltschaniwskyj (2010) gametogenesis follows a seasonal cycle in temperate climates while in tropical zones there are multiple spawnings throughout the year, due to relatively warm water temperatures year round with minimal seasonal change.

1.2 Comparison between River Ecosystems

The results related to the River experiments (**Chapters 2 and 3**) demonstrated that in Mexico a difference between seasons is very clear and this could have an influence in the outcome. Comparing endpoints results alone there is a clear difference among temperate and tropical ecosystems, where evidently the organisms from Minho River (**Chapter 3**) demonstrate a higher adverse effect followed by bivalves from Champoton River in the rainy season (**Chapter 2**). These differences are probably related to the fact that experiments were not performed in the same year, there are different degrees of contamination and the physiology and behavior of clams differ until certain

point.

Comparing PCAs (**Figures 9-12 at Chapter 2; Figures 11-12 at Chapter 3, Supplementary Material**) organisms from Minho River seems to have an overall response more similar to the rainy season of Champoton River. This could be an indication of the influence of abiotic factors more than the presence of contaminants. Besse, Geffard, & Coquery (2012) already postulated the higher degree of difficult to monitor continental waters (streams and rivers) compared to marine and estuarine environments. Due to the strong variability in size, physical-chemical characteristics, tides and seasonal fluctuations it is extremely hard to have a realistic comparison, even when there is proximity in these characteristics (Capela et al., 2016; Ferreira et al., 2007, 2006).

The four common endpoints (AChe, GST, CAT and CI) for the river experiments were considered (**Tables 3 to 5**; **Figures 5 to 10, Supplementary Material**) and it is possible to notice that Champoton in the rainy season has a more clear response, where the groups are distinct. A possible explanation could be that, due to the rain there is a higher presence of contaminants in the river, leading to an adverse effect in the organisms. Runoff of chemicals can vary from 5 to 15% of rainfall (Wauchope et al., 2004) and it was indicated by several authors that increased precipitation enhances runoff contaminated with pesticides (Carere et al., 2011; Delcour et al., 2015; Oliver et al., 2012; Probst et al., 2005; Reilly et al., 2003; Turner et al., 1994). In tropical and developing countries (as México), that will mostly suffer a higher impact due to climate change, the fate and behavior of pesticides will change with increasing temperature, consequently affecting the pesticide use. This is an important fact, since some countries might re-introduce or increase the use of banned or restricted pesticides (Delcour et al., 2015; Macdonald et al., 2005). Studies related to these environments and to pesticides began to have a higher and alarming importance, considering that there will be an impact on the consumer exposure at the end of the food chain.

1.3 Assessment of Laboratory experiments

The laboratory experiments with a model organism as *Mytilus*

galloprovincialis (**Chapters 4 and 5**) show that the response of organisms is more time-related than based just in the concentration used. Chronic exposures could have more influence in the physiology of the organisms, since it is constantly exposed and have no time to recover and restore their healthy status. However sometimes the organisms could be acclimate to this environment and restore some of their health status and be functioning. Some studies (Fernández et al., 2010; Regoli and Principato, 1995) related no variation or a transient response what suggest an adaptive or compensatory mechanism to chronic exposures. This compensatory adaptive response was noticed in experiments with *Austocochlea porcata* (Reid and MacFarlane, 2003) and *Mytilus galloprovincialis* (Fernández et al., 2010) were after some time the organisms acclimate to stress and returned the enzymatic activity close to those of controls exposures. Tolerance to chronic exposure could originate increase allocation of energy and resources to defense mechanisms and allows persistence of xenobiotics in the food webs, which is worrying for those compounds inclined to biomagnification or to toxicity transference between successive links (Amiard-Triquet et al., 2012b).

In the other hand acute or pulse exposures, let the organism to have some kind of recovery, although if the contamination is extreme there is no process of recovery and the oxidative stress affect the lipid peroxidation (LPO), what could cause DNA damage and apoptosis, leading to the death of organism (van der Oost et al., 2003). In our study is possible to see that the detoxification system of mussels is working, their defense mechanisms are resilient and contaminants caused no permanent damage to the physiology of bivalves. The increased or decreased of the enzymatic activity can persist in time after a short-term perturbation, or instead recover to the original status (Proia et al., 2011).

Comparing PCAs with the main four endpoints (AChe, GST, CAT and CI) for the laboratory experiments (**Tables 6 and 7; Figures 11 to 14, Supplementary Material**) with the ones at **Chapter 4** and **5,** it is possible to notice that there is not a big difference in the formation of clusters. These effects were not observed in field exposures, what could be an evidence of seasonal fluctuations and abiotic factors in the response of organisms. In a controlled environment, as the ones performed with mussels, we highlighted

the effect of contaminants per se, an important step to a better understanding of toxicological mechanistic of contaminants in the physiological response of bivalves.

1.4 General Overview

This study is an attempt to better understand the large-scale effects of pollution and other stressors like habitat change on the health status of different bivalve species. Currently, studies that encompass such processes are scarce, especially related to tropical zones, highlighting the relevance of this project in the interpretation of the biological responses of bivalves and, thus, improving the risk assessment processes related to these organisms.

During the reading of this thesis it is possible to notice several points in common, that were pointed out before, of the works developed in Portugal and México. As demonstrate in several other studies (Cravo et al., 2012; He et al., 2011; Liu et al., 2014; Matozzo et al., 2012; Milun et al., 2016) there is a physiological effect associated with organic pollutants, this is expressed by the organisms through enzymatic activities, where is possible to assess the health state of bivalves.

Field studies are each day more utilized to have a better estimative of the organisms' behavior and what could cause deleterious effects. To be able to understand specific effects it is important to link effects found in the laboratory for a species and its adverse effects in a controlled environment, to a natural ecosystem, with all seasonal and annual fluctuations. Usually, laboratory experiments help to identify biomarkers of interest, and the validation with in situ studies help to accurately assess the influence of external environmental factors (temperature, salinity, food availability, seasonality) and internal responses (condition index and biomarkers) (Luna-Acosta et al., 2015). It was expected that bivalve species collected from the wild and used in laboratory have an undervaluation of the risk in field situations, because its tolerance to natural stress could spread to tolerance to chemical stress. However, the contrary has been established, species at the limit of their tolerance to stress are more sensitive to any additional chemical stress (Amiard-Triquet et al., 2011). It is clear in this study that temperature and different ecosystems evokes differences, but even with unique

physiological responses there is a tendency that all organisms follow. Survival is after all the major tool of adaptation for all living organisms.

Comparing the transplants realized in México and Portugal (**Chapters 1 and 2**), it is possible to make some considerations, after a period of adaptation (about 30 days) to the new surroundings all bivalves employed in this kind of study tend to have ways to adapt to their original health state. In general, during the 90 days of experiments is possible to notice gradual recuperation of the activities to a fit state of the organism. Longer periods of transplants would bring even more information about these organisms, also studies comparing transplanted and wild animals have the tendency to avoid the seasonal fluctuations, what is a confounding factor of the physiological responses. With this in mind would be possible to assess in a more clear way the effects of effluents or anthropogenic sources of contamination. However, in the field, incidents such as loss of enclosures during storms or acts of malevolence are still a concern for a higher experimental time (Berthet, 2012).

In the laboratory experiments (**Chapters 5 and 6**) it is possible to notice that after some period of exposure, the organism have the tendency to group together causing to have less and less differences between concentrations. It is possible that the digestive gland and other organs are so damage that the contaminants are not having a great adverse effect, or in the same continuous state of contamination the bivalves have faster mechanisms of detoxification. The digestive gland is the major site of xenobiotic and oxy-radical generating biotransformation enzymes (Livingstone et al., 1992); it is used as energy source during periods of physiological stress (Bayne et al., 1976; Gosling, 2003). According to Lowe & Moore (1979) the digestive gland tissue present the formation of granulocytomas (inflammatory lesions) and tissue breakdown when challenged with contaminants.

1.5 Last Considerations and Future Perspectives

Aquaculture of bivalves is a growing activity due to 53% of commercial marine fish stock is fully exploited (FAO, 2010). Only in 2010, 14 million tons of molluscs were produced, representing 23.6% of world aquaculture production (FAO, 2012; Lacoste and Gaertner-Mazouni, 2015). Moreover aquaculture will be the main source of seafood to human consumption,

creating the need to deeply understand the anthropogenic impact in the life cycles of bivalves. Shallow waters contiguous by cities (due to economic reasons) will be exploited even more for this purpose, however this water-bodies are affected by several sources of contamination (sewages and pesticides, among others), creating a need of better oversight and management of these ecosystems and its biota (Galvao et al., 2012). The first step to achieve a better risk assessment of these areas would be through field studies, several tools (biochemical, physiological, statistical, and behavioral) have been used to assess the environmental risk of contaminants and to establish environmental quality standards favoring biological conservation (Amiard-Triquet et al., 2012a).

Field studies that include the use of multiple biomarkers allow assessment of the impact of several environmental stressors and constitute a sensitive early warning signal of deleterious effect (Lagadic et al., 1994). Active biomonitoring using bivalves has been very successful and nowadays is a recommended tool for environmental monitoring programs (Bebianno et al., 2004; C. Porte, M. Solé, V. Borghi, 2001; Cardoso et al., 2015; Cravo et al., 2012; Luna-Acosta et al., 2015; Romero-Ruiz et al., 2003; Seabra Pereira et al., 2014; Turja et al., 2013; Viarengo et al., 2007). Several endpoints should be selected in any biomonitoring program; as was highlighted through this thesis and other studies (Depledge and Fossi, 1994; Hamza-Chaffai, 2014; Handy et al., 2003; McCarthy and Shugart, 1990) the number of biomarkers can modify the overall response of organisms. PCA analysis showed a more realistic and clear effect when 5 or 8 endpoints were used instead of the four common in all experiments. According to Guerlet (2007) the use of PCA presents several advantages: possible application without any previous information on the gradient of stress, flexibility and the possibility of bringing together biological and physicochemical data without the latter influencing the profile of the PCA. The use of this tool should be applied in further studies to a more comprehensive understanding of the physiology and behavior of organisms, leading to a better comparison and validation of data among different in situ and laboratory experiments.

In recent years the application of genomics, proteomics and metabolomics have been used in the ecotoxicology field (Campillo et al.,

2015; Helmholz et al., 2015; Rocco et al., 2015; Snape et al., 2004; Van Aggelen et al., 2010; Viant, 2007). As pointed out by Vidal-Liñán & Bellas (2013) these new biomarkers provide information about gene expression, protein levels and cell metabolites in organisms exposed to contaminants, what may bring new insights into the mechanistic and toxicity of pollutants, improving the understanding of their environmental risks. The use of these new tools combined with the more traditional biomarkers could help to establish the link between cellular and molecular changes and the effect in individual and/or population levels. These links are called adverse outcome pathways (AOPs) and its use is important for risk assessment and regulatory applications (Groh et al., 2015). AOPs are still a new approach to the mechanistic toxicological data, especially when related to bivalves, and their use in the future should increase, bringing the ecotoxicology field to a new level in the evaluation of contaminants.

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SUPPLEMENTARY MATERIAL

Table 1. PCA: Component loadings of the variables for the two principal components in Términos Lagoon.

Variables	*Component 1*	*Component 2*
Eigen values	*2.982*	*0.856*
% of variance	*74.53*	*21.39*
GST	*0.904*	*-0.417*
CI	*0.878*	*-0.469*
Ache	*0.870*	*0.405*
CAT	*0.799*	*0.546*

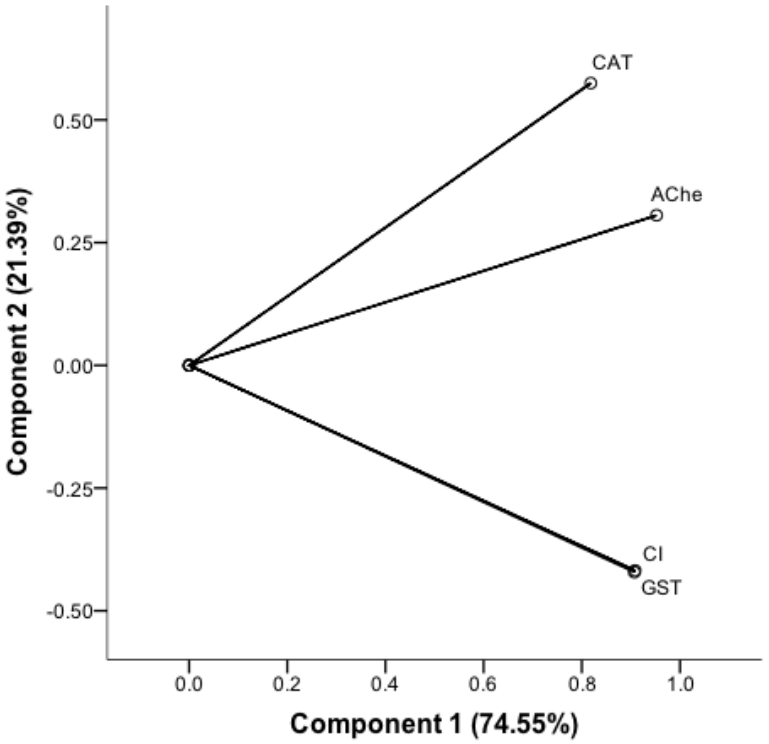

Figure 1. Plot of variable vectors for the two dominant components produced by biomarkers (AChe, GST, CAT) and condition index (CI) of Términos Lagoon.

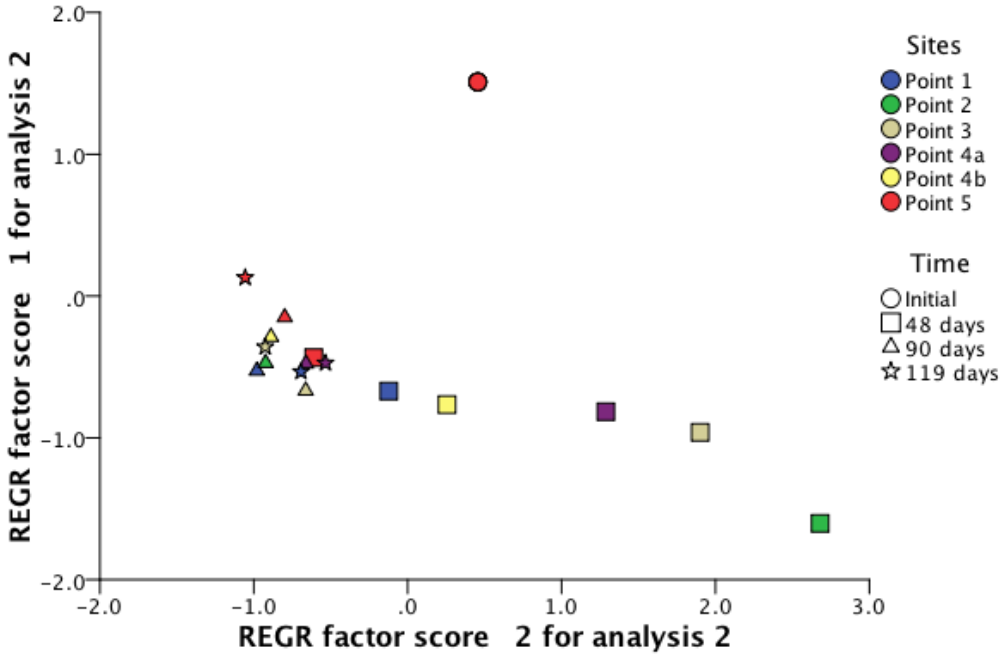

Figure 2. The distribution diagram of the different groups of sites during different experimental periods as a function of the two principal component axis. Principal component loading and total variance associated with each axis are provided in Table 1.

Table 2. PCA: Component loadings of the variables for the two principal components in Aveiro Estuary.

Variables	*Component 1*	*Component 2*
Eigen values	*2.901*	*1.015*
% of variance	*72.53*	*25.382*
CAT	*0.988*	*-*
Ache	*0.984*	*-*
GST	*0.923*	*0.347*
CI	*-0.323*	*0.944*

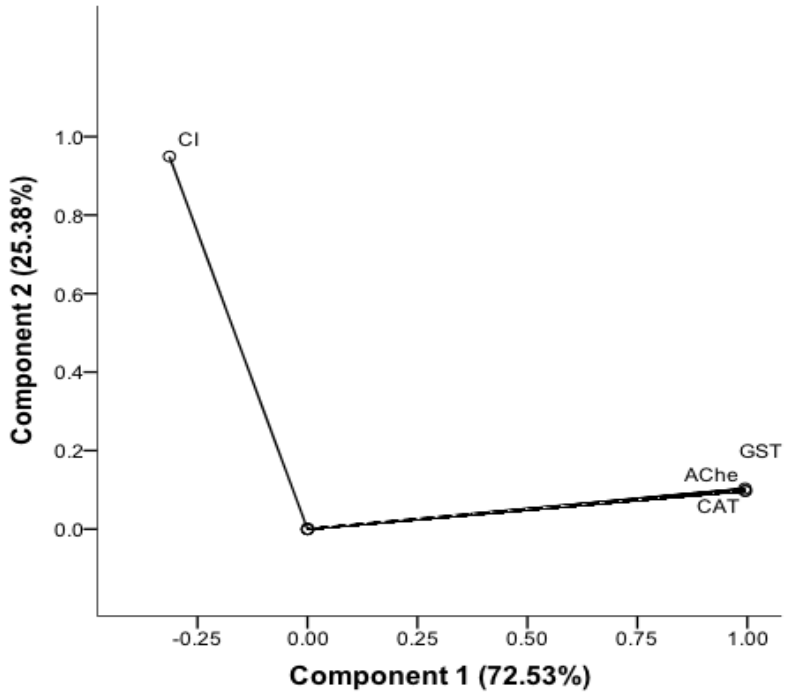

Figure 3. Plot of variable vectors for the two dominant components produced by biomarkers (AChe, GST, CAT) and condition index (C.I.) of Aveiro Estuary.

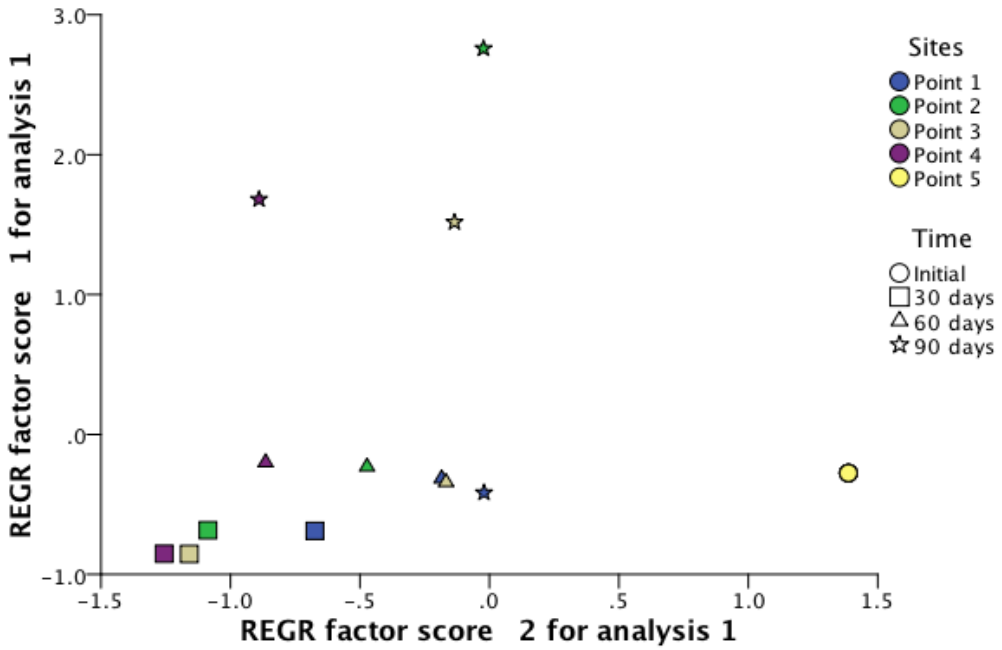

Figure 4. The distribution diagram of the different groups of sites during different experimental periods as a function of the two principal component axis. Principal component loading and total variance associated with each axis are provided in Table 2.

Table 3. PCA: Component loadings of the variables for the three principal components in Champoton River at rainy season.

Variables	*Component 1*	*Component 2*
Eigen values	*1.360*	*1.115*
% of variance	*34.012*	*27.864*
Ache	*0.675*	*-0.416*
CI	*0.610*	*0.492*
CAT	*0.476*	*0.617*
GST	*-0.554*	*0.564*

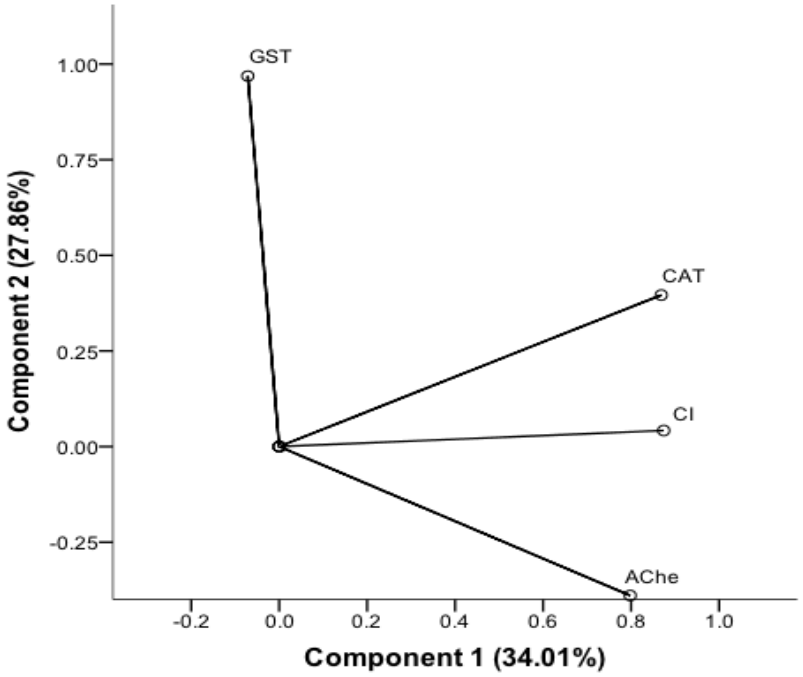

Figure 5. Plot of variable vectors for the two dominant components produced by biomarkers (AChe, GST, CAT, SOD) and condition index (CI) of Champoton River at rainy season.

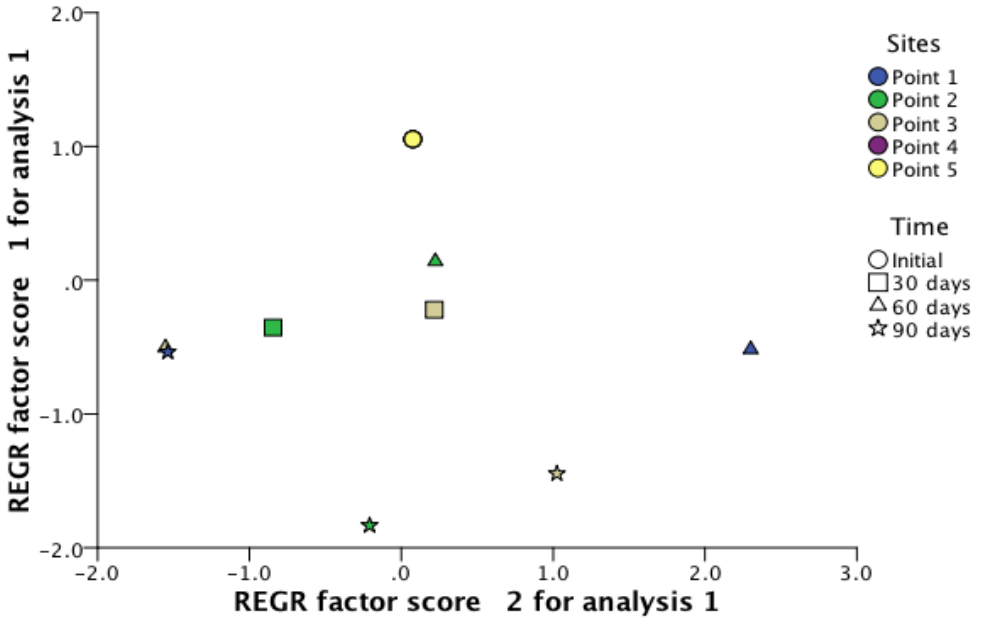

Figure 6. The distribution diagram of the different groups of sites during different experimental periods as a function of the two principal component axis in Champoton River at rainy season. Principal component loading and total variance associated with each axis are provided in Table 3.

Table 4. PCA: Component loadings of the variables for the three principal components in Champoton River at dry season.

Variables	*Component 1*	*Component 2*
Eigen values	*2.020*	*1.067*
% of variance	*50.50*	*26.67*
GST	*0.853*	*0.328*
CI	*0.745*	*-0.438*
Ache	*0.668*	*0.592*
CAT	*-0.540*	*0.646*

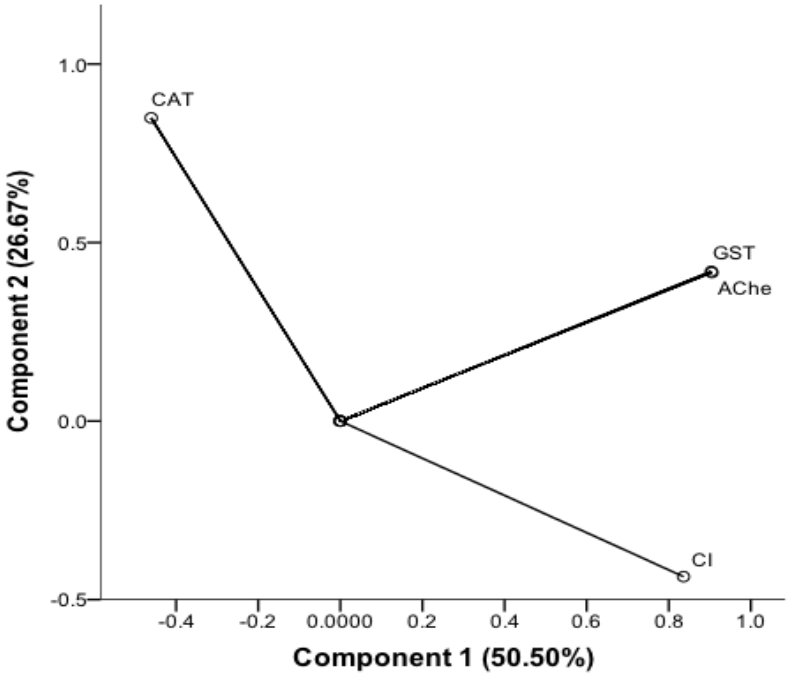

Figure 7. Plot of variable vectors for the two dominant components produced by biomarkers (AChe, GST, CAT) and condition index (CI) of Champoton River at dry season.

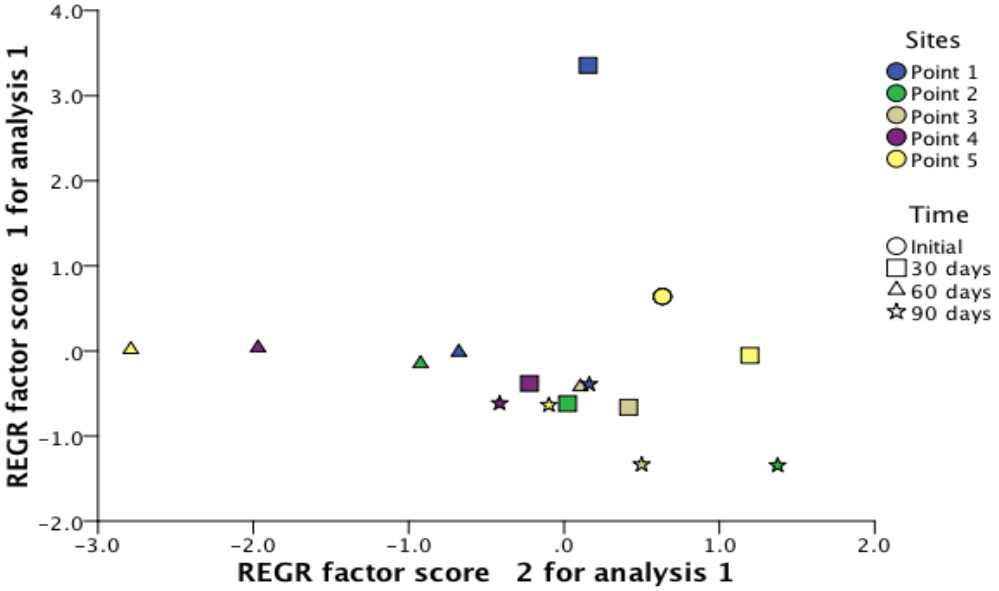

Figure 8. The distribution diagram of the different groups of sites during different experimental periods as a function of the two principal component axis in Champoton River at dry season. Principal component loading and total variance associated with each axis are provided in Table 4.

Table 5. PCA: Component loadings of the variables for the two principal components in Minho River.

Variables	*Component 1*	*Component 2*
Eigen values	*2.050*	*1.123*
% of variance	*51.24*	*28.07*
CI	*-0.904*	*-*
GST	*0.701*	*-*
CAT	*0.584*	*-0.777*
Ache	*0.633*	*0.714*

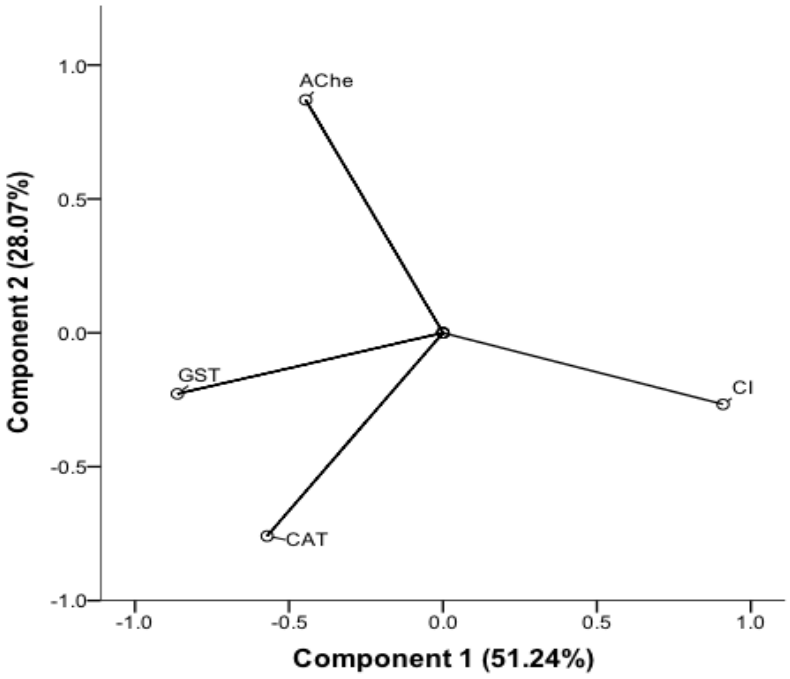

Figure 9. Plot of variable vectors for the two dominant components produced by biomarkers (Ache, GST, CAT, LPO, GR) and condition index (C.I.) of Minho River.

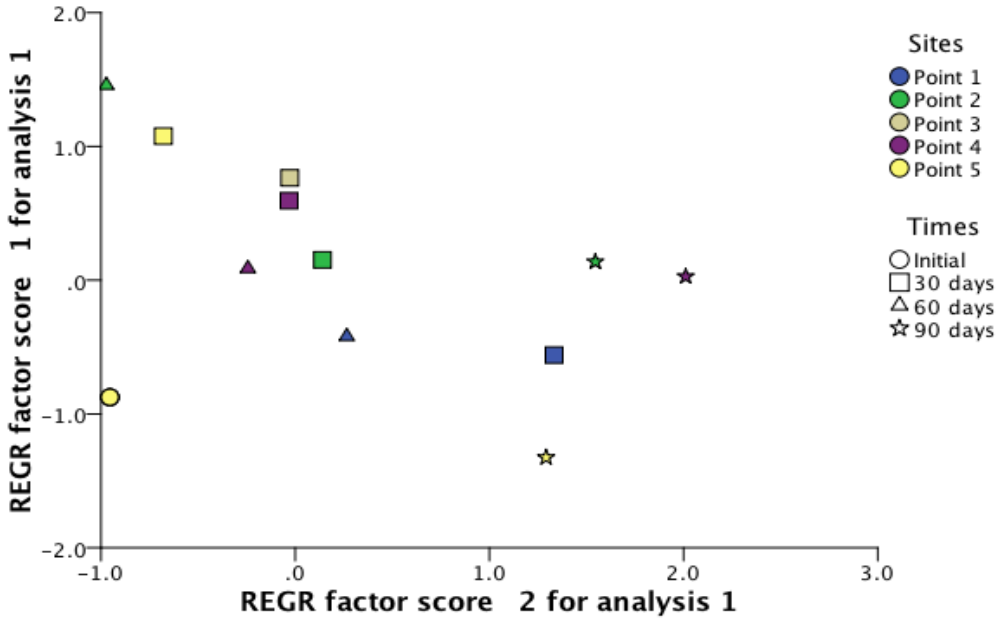

Figure 10. The distribution diagram of the different groups of sites during different experimental periods as a function of the two principal component axis. Principal component loading and total variance associated with each axis are provided in Table 5.

Table 6. PCA: Component loadings of the variables for the two principal components in experiment with *Mytilus galloprovincialis* and benzo(a)pyrene.

Variables	***Component 1***	***Component 2***
Eigen values	*2.586*	*0.596*
% of variance	*64.65*	*14.89*
CAT	*-0.873*	*-*
GST	*0.821*	*0.361*
CI	*0.784*	*-*
AChe	*-0.732*	*0.643*

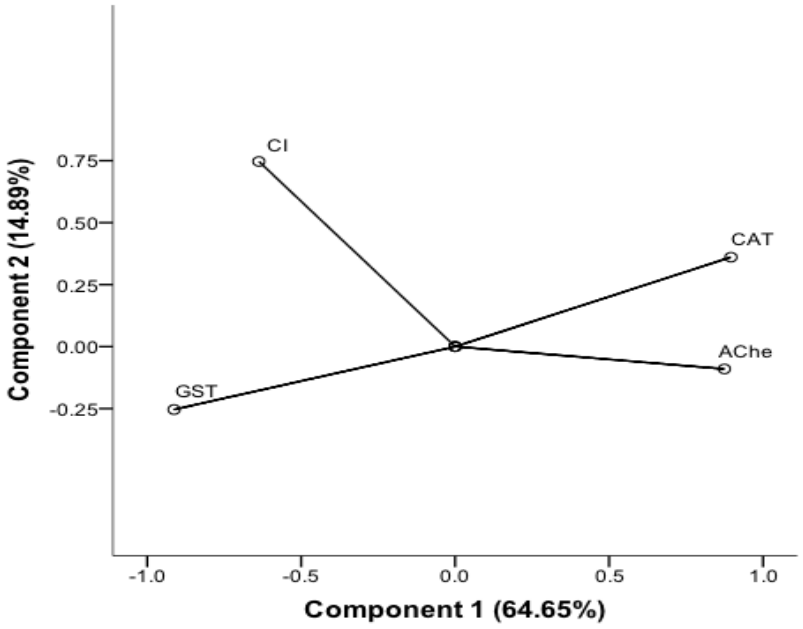

Figure 11. Plot of variable vectors for the two dominant components produced by biomarkers (AChe, BChe, PrChe, GST, CAT, GR, LPO) and C.I. of an exposure with benzo(a)pyrene.

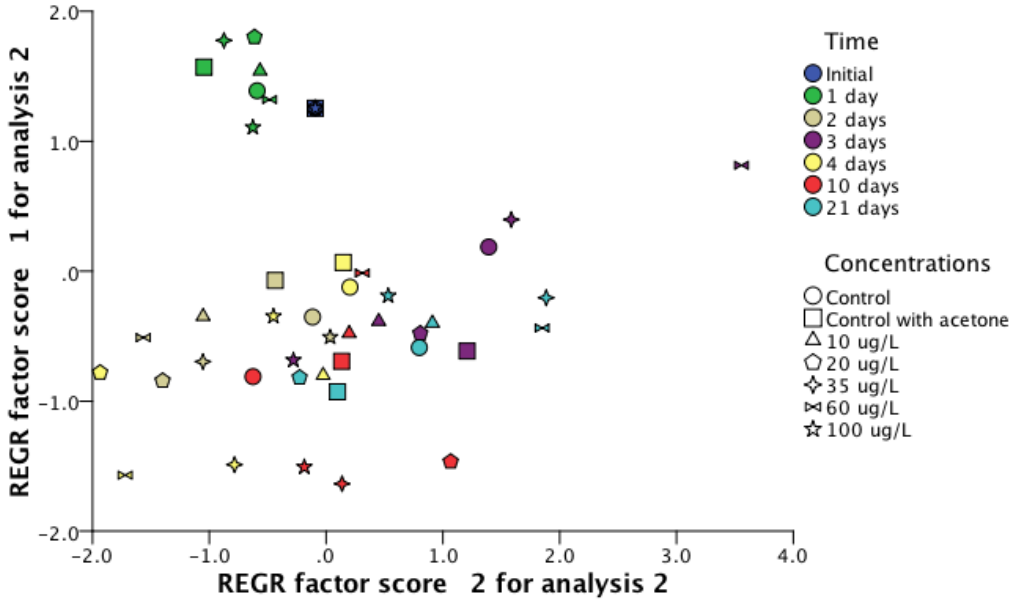

Figure 12. The distribution diagram of the different groups of benzo(a)pyrene concentrations during different experimental periods as a function of the two principal component axis. Principal component loading and total variance associated with each axis are provided in Table 6.

Table 7. PCA: Component loadings of the variables for the three principal components in an experiment with *M. galloprovincialis* and endosulfan.

Variables	*Component 1*	*Component 2*
Eigen values	*1.856*	*1.167*
% of variance	*66.39*	*29.16*
CI	*0.811*	*0.424*
AChe	*-0.791*	*-0.335*
GST	*0.659*	*-0.470*
CAT	*-0.371*	*0.809*

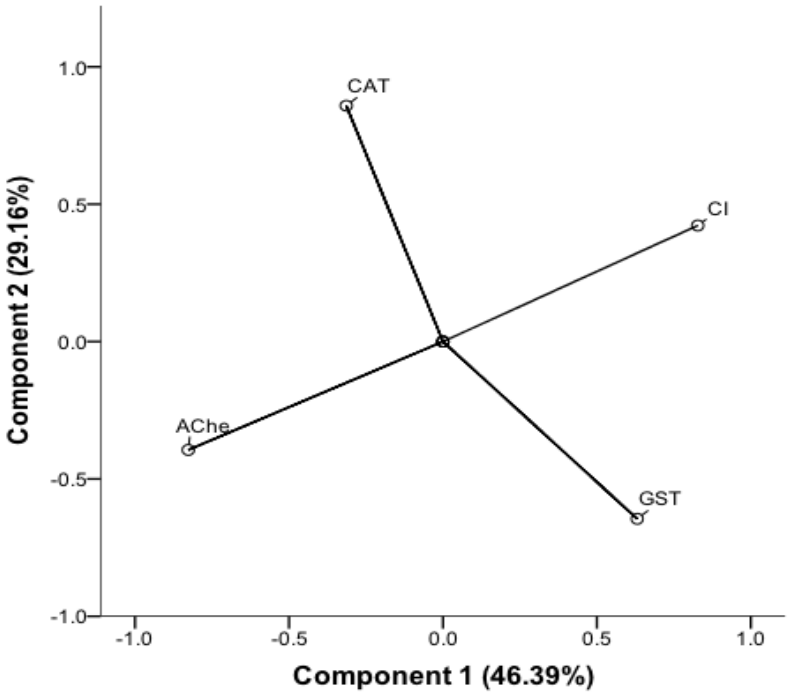

Figure 13. Plot of variable vectors for the two dominant components produced by biomarkers (AChe, BChe, PrChe, GST, CAT, GR, LPO) and C.I. of an exposure with endosulfan.

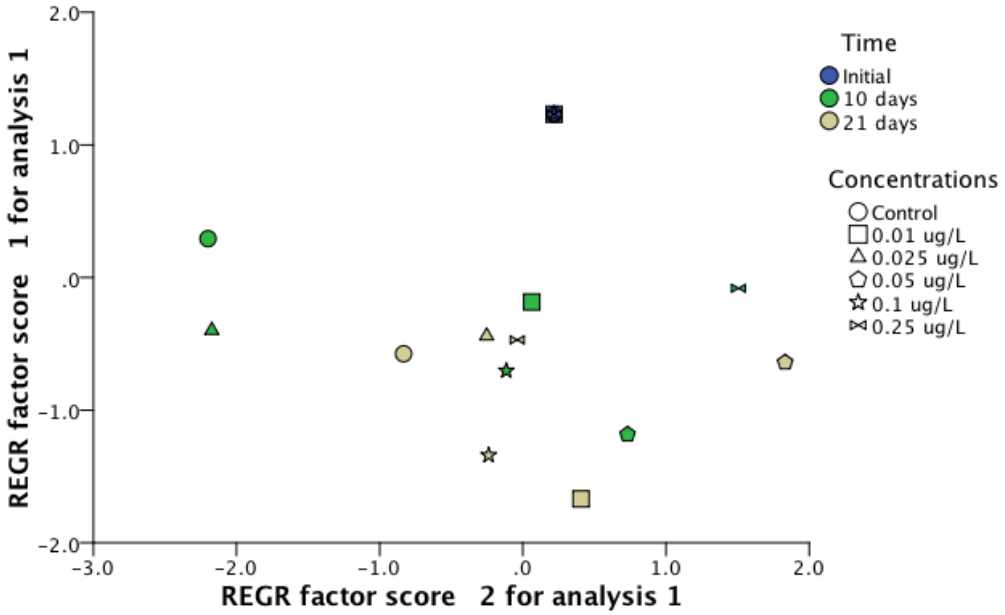

Figure 14. The distribution diagram of the different groups of endosulfan concentrations during different experimental periods as a function of the two principal component axis. Principal component loading and total variance associated with each axis are provided in Table 7.